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 consumption on maternal nutrient intake, fatty acid status and immunity

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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SALMON IN PREGANCY STUDY (SIPS): THE EFFECTS OF INCREASED OILY FISH CONSUMPTION ON MATERNAL NUTRIENT INTAKE, FATTY ACID STATUS AND IMMUNITY

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The prevalence of childhood atopic diseases (eczema, asthma, allergies, hay-fever) has increased during the last 30 years. Epidemiological studies link higher fish intake during pregnancy with lower risk of atopy in the offspring. Oily fish provide the long chain (LC) n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as vitamin D and antioxidants (selenium). Fish oil provides EPA and DHA and fish oil supplementation during pregnancy alters offspring immunity in a way that would be consistent with lowered risk of atopy. There are no studies of oily fish intervention in pregnancy.

The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial of oily fish intervention during pregnancy. The main outcome measures of SIPS were the clinical signs of atopy in the offspring (not reported here). The current thesis presents and discusses results of SIPS mainly relating to the mother. The hypotheses examined here are that increased oily fish consumption during pregnancy will: a) increase maternal LC n-3 PUFA intake b) increase maternal LC n-3 PUFA status, c) alter maternal immunity, which may influence the developing foetal immune system in a way that would decrease atopy risk for the offspring.

Pregnant women (n = 123) with high risk of having atopic offspring and with low habitual intake of oily fish (≤ 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 women attended a clinic at weeks 20 (n = 123), 34 (n = 110), and 38 (n = 91) of pregnancy at which fasting blood samples were collected for fatty acid and immunological analysis, and a food frequency questionnaire (FFQ) was administered (at 20 and 34 weeks). At delivery, placenta and umbilical cord tissue were collected for fatty acid analysis. Mothers were followed-up at 3 months postpartum when the FFQ was administered (n = 88). Maternal plasma, peripheral blood mononuclear cell (PBMC), placenta nad umbilical cord tissue fatty acid compositions were determined by gas chromatography (GC). Maternal immune cell subsets were determined by flow cytometry (FACS); ex-vivo cytokine production by PBMC in response to stimulants (allergens, mitogen, and toll-like receptor (TLR) ligands) was determined by cytometric bead array (CBA) and FACS; and eicosanoid (prostaglandin (PG) E_2) production by PBMC was determined by enzyme-linked immunosorbent assay (ELISA).

Subjects complied with the salmon intervention and this increased their intakes of EPA, DHA, vitamin D and selenium. The salmon intervention prevented the pregnancy-associated depletion in LC n-3 PUFA and resulted in higher LC n-3 PUFA status in maternal plasma, maternal PBMC, placenta and cord tissue. Effects of pregnancy on many of the immune parameters assessed here were identified. However, the salmon intervention had only limited impact on maternal immunity as measured here, and thus it cannot be concluded whether the intervention would have an effect on the immune system of the offspring.

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DECLARATION OF AUTHORSHIP

I, Lefkothea-Stella Kremmyda, declare that the thesis entitled:

Salmon in Pregnancy Study (SIPS): The effects of increased oily fish consumption on maternal nutrient intake, fatty acid status and immunity

and 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 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;
- where I quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- food frequency quenstionnares (FFQ) were administered by myself in collaboration with Maria Vlachava (PhD candidate). FFQ data were entered by Ken Cox (Medical Research Council, Southampton) who calculated frequency and nutrient intakes according to the SWS database. Fish diaries were data entered and analysed by myself in collaboration with Maria Vlachava.
- I performed all of the following methods: fatty acid analysis of plasma (all fractions), immune cells, placenta and cord tissue, whose results were reported in this thesis, for the largest subset of the samples collected. One subset was analysed by Maria Vlachava (PhD candidate);
- I performed all of the following methods: immune cell phenotyping (flow cytometry), immune cell culture with various stimulants, cytokine determination (CBA), prostaglandin E_2 measurement (ELISA), whose results were reported in this thesis, for the largest subset of the samples collected. One subset was analysed by Maria Vlachava (PhD candidate), Dr Paul S. Noakes and Dr Elizabeth A .Miles;
- all statistics and figures presented in the present thesis were generated by myself, after receiving consultation from the statisticians Dr Scott Harris and Dr Sarah Crozier;
- parts of this work (section 1.5) have been published as:

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Signed:

Date:

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Abbreviations

AHR	Airway hyper-responsiveness
ANOVA	Analysis of variance
APC	Antigen presenting cell(s)
ARA	Arachidonic acid
CAPS	Childhood Asthma Prevention Study
CBA	Cytometric bead array
CE	Cholesteryl ester(s)
CETP	Cholesteryl ester transfer protein
CI	Confidence interval
COMA	Committee on Medical Aspects of Food Policy
Con A	Concanavalin A
COX	Cyclooxygenase
DAG	Diacylglycerol
DC	Dendritic cells
DDT	Dichloro diphenyl trichloroethane(s)
Der p 1	*Dermatophagoides pteronyssinus*
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DHT	Delayed-type hypersensitivity
Dl	Dioxin like
DMSO	Dimethyl sulphoxide
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid(s)
ELISA	Enzyme-linked immunosorbent assay
EPA	Eisocapentaenoic acid
FA	Fatty acid(s)
FACS	Flow cytometer
FAME	Fatty acid methyl ester(s)
FFQ	Food frequency questionnaire(s)
FID	Flame ionisation detector
FITC	Fluorescein isothiocyanate isomer 1
FO	Fish oil
FSC	Forward scatter
GC	Gas chromatography
HDL	High density lipoprotein(s)
HDM	House dust mite
HEP	Histamine Equivalent in skin Prick testing
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HI-FBS	Heat-inactivated foetal bovine serum
HLA	Human leukocyte antigen
HPEPE	Hydroperoxyeicosapentaenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN	Interferon

Ig	Immunoglobulin
IHN	Institute of Human Nutrition
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
LC	Long chain
LDL	Low density lipoprotein(s)
LH	Lithium heparin
LOX	Lipoxygenase
LPL	Lipoprotein lipase(s)
LPS	Lipopolysaccharide
LRNI	Lower Reference Nutrient Intake
LT	Leukotriene(s)
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MTHF	Methyl-tetra-hydrofolic acid
MUFA	Monounsaturated fatty acid(s)
n-3	omega 3
n-6	omega 6
NDNS	National Diet and Nutrition Survey
NEFA	Non-esterified fatty acid(s)
NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drugs
NSB	Non-specific binding
OR	Odds ratio
Ova	Ovalbumin
p	*p*-value
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PC	Phospholipid(s)/ Phosphatidylcholine
PCA	Principal component analysis
PCB	Polychlorinated biphenyl(s)
PE	Phycoerythrin
PG	Prostaglandin(s)
PGN	Peptidoglycan
PHA	Phytohaemaglutinin
Poly I:C	Polyinosine-polycytidylic acid
PTWI	Provisional tolerable weekly intake
PUFA	Polyunsaturated fatty acid(s)
RCT	Randomised controlled trial(s)
RNI	Reference Nutrient Intake
SACN	Scientific Advisory Committee on Nutrition
Sal s1	Salmon paralbumin
sd	Standard deviation
Se	Selenium
SFA	Saturated fatty acid(s)
SIPS	Salmon in Pregnancy Study
SPE	Solid phase exctraction
SPSS	Statistical Package for Social Sciences

SPT	Skin prick testing
SSC	Side scatter
SWS	Southampton Women's Survey
SWSIPS	Southampton Women's Survey/ Salmon in Pregnancy Study
TAG	Triglyceride(s)
TCR	T-cell receptor(s)
TGF	Transforming growth factor
Th	Helper T-cell(s)
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(s)
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein(s)
vs.	Versus
WHO TEQ	World Health Organisation toxic equivalent
χ^2	Chi-square

1 General Introduction

This introduction aims to provide information on fatty acids (Section 1.1), the immune system (Section 1.2), immune system changes during pregnancy and the early life origins of atopic disease (Section 1.3), the link between fatty acids and the immune system (Section 1.4), the scientific evidence available concerning the effects of increased intake of long-chain n-3 fatty acids during early life on immune system development and atopic disease (Section 1.5), leading to the hypotheses and aims of this PhD thesis (Chapter 2).

1.1 Fatty Acids: Structure, Nomenclature, Types, Dietary Sources, and Metabolism

The aim of this section is to provide introductory information about fatty acids including their nomenclature, structure, and metabolism, as well as dietary sources and intakes and current recommendations.

1.1.1 Structure of fatty acids

Lipids are organic compounds composed of a carbon skeleton with mainly hydrogen and oxygen substitutions, and include fatty acids and their esters with alcohols such as glycerol or cholesterol.

Fatty acids are the main components of dietary lipids. They are carboxylic acids, varying in chain length from one to more than 30 carbons. Their structure is RCOOH, where R can be a hydrogen in formic acid, a methyl group (CH_3) in acetic acid, or a longer hydrocarbon chain of up to more than 30 methylene (CH_2) groups and a terminal methyl group. The absence of double bonds in the hydrocarbon chain of a fatty acid defines it as saturated, whereas an unsaturated fatty acid contains one or more double bonds in the hydrocarbon chain. Fatty acids with one double bond in the hydrocarbon chain are termed monounsaturated fatty acids (MUFA) and those with two or more double bonds in the hydrocarbon chain are termed polyunsaturated fatty acids (PUFA).

1.1.2 Physicochemical properties of fatty acids

In general, saturated fatty acids 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, PUFA are usually more easily liquefied than saturated fatty acids or MUFA. Unsaturation results in structural variety in fatty acids and allows for isomerisation. A saturated fatty acid occupies, in general, less space than an unsaturated fatty acid of the same length. This is because the insertion of double bounds 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 as each other the bond is termed *cis*-unsaturated and when the hydrogens are on the opposite side of the double bond to one another 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 saturated fatty acid. Lastly, most naturally occurring PUFA 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 particular metabolic processing of fatty acids or of industrial processing of fats and oils for use in the food industry. These isomers are termed conjugated.

1.1.3 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 PUFA, 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 1.1 presents the nomenclature and dietary sources of common fatty acids.

Table 1.1 Nomenclature and typical sources of fatty acids

Systematic name	Common name	Shorthand notation	Typical sources
Saturated fatty acids			
Dodecanoic	Lauric	12:0	Coconut oil
Tetradecanoic	Myristic	14:0	Milk
Hexadecanoic	Palmitic	16:0	Milk, eggs, animal fats, meat, 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
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	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, 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

Modified from (1)

1.1.4 Synthesis and metabolism of fatty acids

Fatty acids can also be categorised into general groups according to the length of their chain. Short-chain fatty acids contain less than 8 carbons, medium-chain fatty acids contain 8-14 carbons, and fatty acids with more than 14 carbons are termed long-chain and are the main constituents of dietary fat.

Long-chain fatty acids can either be taken directly from the diet, or can originate by complete synthesis from acetyl-coenzyme A in the cytosol, or by chain elongation of a pre-existing shorter chain fatty acid. Saturated fatty acids and most MUFA can be synthesised in mammals from non-fat precursors, such as glucose or amino acids.

However, not all fatty acids can be synthesised *de novo* (from acetate) in mammals, as they cannot insert double bonds before carbon 9 in oleic acid. More specifically, mammals cannot convert oleic acid (18:1n-9) into linoleic acid (18:2n-6), or linoleic acid into α-linolenic acid (18:3n-3). Linoleic and α-linolenic acids are the primary dietary *cis*-PUFA and, because they cannot be synthesised *de novo*, their intake from food sources is essential. Thus linoleic and α-linolenic acids are termed essential fatty acids. What is more, mammalian tissues cannot interconvert n-6 and n-3 fatty acids. Only plants are capable of these conversions.

However, elongases and desaturases in mammalian cells are able to extend and insert further double bonds into linoleic and α-linolenic acids taken from the diet, these fatty acids acting as precursors of the families of n-3 and n-6 fatty acids. In particular, docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) can be made by elongation and desaturation of α-linolenic acid, and arachidonic acid (ARA; 20:4n-6) can be made by elongation and desaturation of linoleic acid. Derivatives of the essential fatty acid metabolism may be described as conditionally essential. The structures of n-3 and n-6 PUFA are presented on Figure 1.1.

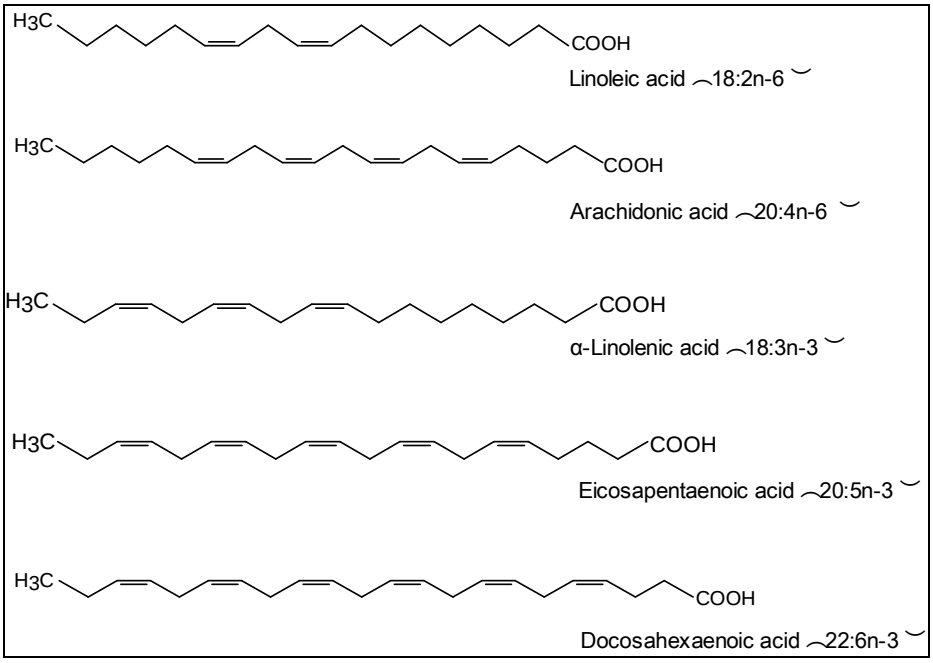


Figure 1.1 Structure of n-3 and n-6 polyunsaturated fatty acids

Figure 1.2 describes the metabolism of n-6 and n-3 polyunsaturated fatty acids. These metabolic pathways are not so efficient in mammals (2), although reasons for this are not yet clear. The two families of fatty acids (n-3 and n-6) have different affinities for the desaturation and elongation enzymes. The metabolic pathway is principally based in the endoplasmic reticulum and has also been demonstrated to occur in mitochondria. The last step of the pathway is more complex, since the existence of Δ4-desaturase in animals has not been proven, and it appears that fatty acids undergo a second elongation, Δ6-desaturation and a final chain shortening in peroxisomes (3, 4).

Linoleic acid is the predominant PUFA in the diet and in the body, accounting for 12-15% of adipose tissue fatty acids (5, 6). In cell membranes, linoleic and arachidonic acids are the principal n-6 PUFA, and DHA is the principal n-3 PUFA (3, 7).

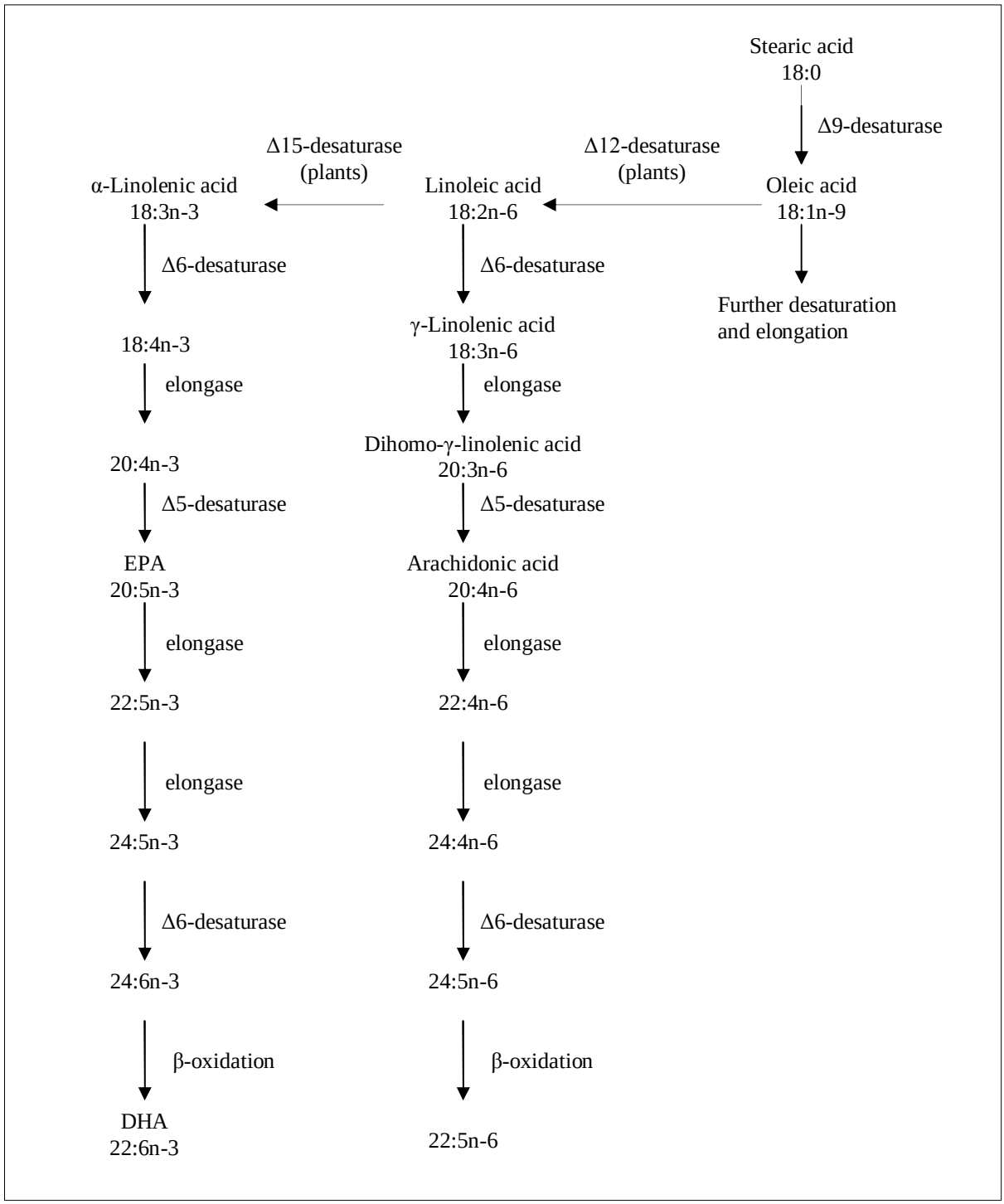


Figure 1.2 Metabolism of n-3 and n-6 polyunsaturated fatty acids
EPA, eicosapentaenoic acid; DHA, docosahexanenoic acid

1.1.5 Sources of n-6 and n-3 fatty acids

Linoleic and α-linolenic acids are found in plant tissues and plant oils. Linoleic acid comprises over 50%, often up to 80%, of the fatty acids found in corn, sunflower, safflower and soybean oils. Other plant oils, such as rapeseed and soybean oil, also contain α-linolenic acid but in smaller amounts (between 5 and 15% of total fatty acids). Flaxseed oil contains a high proportion of α-linolenic acid. Table 1.2 describes the linoleic and α-linolenic acid contents of various plant oils, seeds and nuts.

Table 1.2 Linoleic and α-linolenic acid content of oils, seeds and nuts

	Polyunsaturated fatty acids, g per 100 g total fatty acids[1]	
Food	**α-Linolenic acid** (18:3n-3)	**Linoleic acid** (18:2n-6)
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
Soyabean oil	7.30	51.50
Sunflower oil	0.10	63.20
Walnut oil	11.5	58.40
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

[1] Data taken from (8)

The long-chain n-3 PUFA EPA and DHA are found in oily fish and fish oils. 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 diet, stage of maturation, season and breeding cycle, after breeding the fat content falls considerably. Oily fish contain 5-20% fat in their flesh 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 an oily fish. However, unlike other canned oily fish, canned tuna is not regarded as oily, as processing of tuna during canning reduces its fat content. Table 1.3 lists common oily and non-oily fish and their n-3 and n-6 PUFA contents.

Table 1.3 Total fat and polyunsaturated fatty acids of fish and fish products

	Total fat and polyunsaturated fatty acids, g per 100 g fish[1]									
Fish	**Total Fat**	Total *cis* **PUFA**	Total **n-3** PUFA	Total **n-6** PUFA	**EPA** (20:5n-3)	**DPA** (22:5n-3)	**DHA** (22:6n-3)	**α-Linolenic** (18:3n-3)	**Linoleic** (18:2n-6)	**ARA** (20:4n-6)
Oily Fish										
Herring, fresh[2]	13.2	2.17	1.83	0.32	0.51	0.11	0.69	0.18	0.29	0.04
Kippers, fresh[2]	17.7	3.86	3.52	0.24	1.15	0.10	1.34	0.24	0.18	0.03
Mackerel, fresh[2]	16.1	3.27	2.78	0.51	0.71	0.12	1.10	0.22	0.30	0.07
Salmon, fresh[3]	11.0[5]	3.10[5]	2.70[4]		1.20	0.20	1.30			
Salmon, canned in brine[2]	7.8	2.24	1.85	0.27	0.55	0.14	0.86	0.08	0.13	0.11
Sardines, canned in oil, drained[2]	14.1	4.94	2.27	2.60	0.89	0.10	0.82	0.36	2.54	0.04
Sprats, fresh[2]	11.0	3.04	2.68	0.31	0.93	0.10	1.35	0.08	0.11	0.07
Trout, rainbow, fresh[2]	5.2	1.73	1.32	0.41	0.23	0.09	0.83	0.06	0.34	0.03
Tuna, fresh[3]			1.50[4]		0.30	0.10	1.10			
Non-oily Fish										
Cod, fresh[2]	0.7	0.28	0.26	0.02	0.08	0.01	0.16	Tr	Tr	0.02
Haddock, fresh[2]	0.6	0.20	0.17	0.03	0.05	0.01	0.10	Tr	0.01	0.01
Plaice, fresh[2]	1.4	0.35	0.32	0.01	0.16	0.04	0.10	0	0	0
Tuna, canned in brine, drained[2]	0.6	0.21	0.17	0.04	0.02	0.01	0.14	0	0.01	0.02
Fish products and Fish dishes										
Fish cakes[2]	3.9	0.93	0.33	0.60	0.05	0.01	0.09	0.18	0.58	0.01
Fish fingers, cod[2]	7.8	1.38	0.17	1.28	0.04	0	0.03	0.10	1.21	0
Scampi, breaded[2]	0.90	0.20	0.06	0.14	0	0	0	0.06	0.14	0
Taramasalata[2]	52.9	15.14	5.00	10.81	0.14	0.05	0	4.43	10.52	0
Crustacea and Molluscs										
Crab[2]	5.50	1.36	1.10	0.24	0.47	0.08	0.45	0.02	0.02	0.12
Mussels[2]	2.70	0.76	0.68	0.08	0.41	0.02	0.16	0.02	0.02	0.05
Oysters[2]	1.30	0.41	0.37	0.05	0.14	0.02	0.16	0.01	0.02	0.01
Prawns[2]	0.60	0.13	0.11	0.03	0.06	Tr	0.04	0	0.01	0.01
Squid[2]	1.70	0.61	0.45	0.16	0.13	0.01	0.29	0	0.12	0.01

[1] PUFA, polyunsaturated fatty acid(s); EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; Tr, traces.

[2] Data taken from (9)

[3] Data taken from (10) cited in (11)

[4] Figure reflects total long chain (LC) n-3 PUFA content, not total n-3 PUFA as in other fish types

[5] Data taken from (8)

1.1.6 PUFA and fish consumption in the UK

Typical adult intakes of linoleic and α-linolenic acids in the UK are 10-15 and 0.75-1.5 g/day respectively (12). The NDNS data (collected in 2000-2001) for adults aged 19-64 years (in the total of the population, including non-consumers of fish) show that mean consumption of total fish is 217 g/week (about 1.55 portions/week), of which white (including fish products and dishes) was 103 g/week and oily fish (excluding canned tuna) 50 g/week (about 0.36 portion/week) (12). Amongst those consuming fish, mean consumption of total fish was 304 g/week, of which white fish and oily fish was 221 g/week and 194 g/week, respectively. The fish mainly consumed were canned tuna (27%), fresh cod (25%), fresh haddock (9%), fresh salmon (9%), and canned and smoked salmon (8%). Only a very small percentage of the fish consumers consumed sardines, pilchards, mackerel, trout, herring and fresh tuna. During the period of the NDNS survey 74% of the participants did not consume oily fish (excluding canned tuna), 65% did not consume coated and/or fried white fish and 82% did not consume other white fish and their dishes. The NDNS data were collected based on a seven day dietary record (12).

The results of the most recent NDNS (2008/2009) showed similar results. Women aged 19-64 years (including non-consumers) consume on average 7 g/day (49 g/week) coated and fried white fish, 15 g/day (105 g/week) other white fish, shellfish and tinned tuna, and 11 g/day (77 g/week) of oily fish. The same report, showed that amongst female consumers of oily fish (29% of women) aged 19-64 years old daily consumption was 39 g/day (273 g/week or 2 portions/week) (13). This data shows that over 70% of women aged 19-64 years do not consume any oily fish, although within the 30% of consumers consumption is in accordance with the minimum recommendation.

The most recent NDNS data (13) did not report on intake of LC n-3 PUFA. However, the previous NDNS results (12) showed that mean intake of EPA plus DHA for adults in the UK was 244 mg/day from fish and animal sources (12). Since about 70% of the adult population do not eat oily fish, this is an overestimate of true average intake which is likely to be 100 mg/day (14) or less (15). Also, according to NDNS (2008/2009) 2% of the population aged 19-64 years is vegetarian (13). However, it has been previously shown that the percentage of women in the UK who are vegetarians may be as high as 12% (12). Exclusion of meat and fish can result in low intake of EPA and DHA among pregnant vegetarian women. The intake of DHA among pregnant vegetarian women has been estimated to be around 10-30 mg/day (16, 17).

1.1.7 Recommendations for dietary intake of LC n-3 PUFA and fish

It is recommended that for infants, children and adults linoleic acid should provide at least 1% of total energy and α-linolenic acid at least 0.2% of total energy (18). The current recommendation for EPA and DHA intake is at least 450 mg/day (11). Also, it has been recommended that DHA intake should be at least 200 mg/day in women of reproductive age (19). To achieve this level of intake, the Committee on Medical Aspects of Food Policy (COMA) recommended eating at least two portions of fish, of which one should be oily, weekly (11). The average fish portion size for adults is defined 140 g, and the estimate used for long-chain n-3 PUFA (EPA and DHA) content of an average oily fish is 1.5-2 g/100 g and for an average white fish 0.3 g/100 g. The long-chain n-3 PUFA content of shellfish is about 0.4 g/100 g (12).

The current recommendation for fish consumption for pregnant women and women of childbearing age is the same as the recommendation given for the general population. However, pregnant women and women of reproductive age should not consume more than a total of two portions of oily fish and tinned tuna per week because of the possible high levels of mercury in these fish. For the same reason, pregnant women are advised to avoid eating shark, marlin and swordfish (11).

The demand for long-chain PUFA for the development of the mammalian brain is high. Preformed long-chain PUFA, particularly DHA and EPA, may need to be provided in the diets of infants to meet the high requirements of rapidly growing tissues and organs (11). The mother is the primary source of these fatty acids for the foetus and breast-fed infant. The current mean dietary intake of DHA in the UK can be estimated around 100 mg/day for adult women, and for those individuals who do not consume fish, or who are consuming a low fat diet the intake is likely to be less (12). It has been recommended that pregnant women need to consume 300 mg DHA/day (20). Koletzko *et al.* (21) concluded that pregnant and lactating women should aim to achieve an average dietary intake of at least 200 mg DHA/day. Intakes of up to 1 g DHA/day or 2.7 g/day long-chain n-3 PUFA have been used in randomised clinical trials in pregnant women without significant adverse effects. According to data reviewed by the Scientific Advisory Committee on Nutrition in 2004 (11), there is some evidence that for many women there is a marginal status for n-3 PUFA during pregnancy and lactation. However, there is a lack of agreed markers which are suitable for defining n-3 PUFA status. The markers usually reported relate to concentrations of fatty acids within the circulation. They may be expressed as total fatty

acids or related to their concentrations in different circulating pools. Concentrations of fatty acids in maternal or umbilical blood have been used as measures of n-3 PUFA status during pregnancy. These measures have been related to maternal dietary intake (22), as well as to pregnancy outcomes (gestation duration, infant growth) (23-26). What is more, during pregnancy a woman must also meet the additional demands related to the accretion of maternal, placental, and foetal 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 PUFA, or specifically DHA, to improve pregnancy outcome needs to be demonstrated (11)

1.2 The Immune System

The aim of this section is to present an overview of the components and the activities of the immune system. The text books 'Immunology' by Roitt, Brostoff, Male (27), and 'Immunobiology' by Janeway (28) are recommended for further reading.

Immunity is the defence system of the body, acting to protect the host from pathogenic agents in the environment, such as bacteria, viruses, fungi and parasites, and other potential insults. It consists of complex pathways of recognition, response, elimination and memory, and it is divided into the innate (natural) and the acquired (specific or adaptive) immune system (28). 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 (27).

1.2.1 Innate Immunity

Innate immunity has no memory and thus is not dependent on prior exposure to an infectious agent. It is the first line of defence against pathogens. The components of the innate immune system are the physical barriers of the body (skin, mucous membranes, lysozyme, stomach acid, commensal bacteria), soluble mediators (macrophage derived cytokines), circulating molecules (complement), and phagocytic cells (granulocytes (neutrophils, basophils, eosinophils), monocytes, macrophages), and natural killer cells. Granulocytes, monocytes and macrophages are involved in pathogen killing, in clearing up cellular and tissue debris, and in tissue repair (29). Phagocytic cells are the main cellular component of the innate immune system. They express on their surface receptors specific for bacterial surface antigens or structures. Phagocytosis is triggered by the binding of the antigen or structure to its receptor. The pathogenic microorganism can then be destroyed by complement or by toxic substances (superoxide radicals, hydrogen peroxide) (30). Bacterial lipopolysaccharide (LPS; known also as endotoxin) is an important exogenous trigger. LPS is a component of the cell wall of Gram-negative bacteria. The role of natural killer cells is also important as they also have receptors on their surface and release cytotoxic proteins against microorganisms (31).

1.2.2 Acquired Immunity

Acquired immunity distinguishes a pathogenic agent as foreign to the host by specifically recognising antigens derived from the pathogen. The components of the acquired 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 are the main cellular component of the acquired immune system and are divided into T- and B-lymphocytes. Before being released into the bloodstream, B-lymphocytes develop and mature in the bone marrow, whereas T-lymphocytes undergo maturation in the thymus. After they are released into the circulation, lymphocytes enter peripheral lymphoid organs: lymph nodes, the spleen, mucosal lymphoid tissue, tonsils, and gut-associated lymphoid tissue. Lymphoid organs are organised to promote the interaction of cells and pathogens, and thus immune responses occur mainly in these organs (29).

The characteristics of the acquired immune response are that it is specific, diverse, has memory, and is self-regulated (32). The specificity of acquired immunity is attributed to the fact that each lymphocyte carries surface receptors specific for a single antigen. The acquired immune system is characterised by diversity since lymphocytes recognise an extremely wide variety of antigens (approximately 10^{11}). As a result, a small number of lymphocytes are able to recognise a specific antigen. For this reason the immune system has developed immunologic memory through clonal expansion (33). A single lymphocyte, after interacting with a specific antigen, proliferates giving rise to a clone of lymphocytes which are able to recognise and destroy the source of this antigen. The effectiveness of the acquired immune system becomes evident over several days after initial activation, and also persists some time after removing the antigen that triggered the response. Immunologic memory results in higher effectiveness and flexibility of the immune system in case of re-exposure to the same antigen. The last feature of the acquired immune system, self-regulation, reflects its ability to re-establish homeostasis through communication between cells (7).

1.2.3 B-Lymphocytes and humoral immunity

B-lymphocytes produce antibodies, which are immunoglobulins that are specific for a single antigen. This form of immunity, which is performed exclusively by B-lymphocytes and deals with extracellular pathogens, is termed humoral immunity. The antibodies on the surface of the B-lymphocyte bind with their specific antigen and this causes B-lymphocytes to proliferate and to differentiate into plasma cells, which in turn secrete large amounts of specific antibodies (33).

Immunoglobulins are proteins consisting of two identical heavy chains and two identical light chains. There are five major classes of immunoglobulin (IgA, IgD, IgG, IgM and IgE) and five types of heavy chain respective to each class of immunoglobulins. Antibodies can bind to microogranisms or toxins and prevent them from attaching to host cells. Also, antibodies activate complement proteins in plasma which promote phagocytosis of bacteria. Antibodies enable opsonisation, the process through which antigens and phagocytes interact. Antibodies possess binding sites for both the antigen and the receptors on the surface of phagocytic cells, and thus act like a link between these two. The antibody class determines the type of the phagocytic cell that it will bind with. Eosinophils are specific for IgE, and macrophages and neutrophils are specific for IgM and IgG. Because of the fact that antibodies are produced through very specific pathways, but promote the response of the innate immune system to destroy the pathogen, they form a bridge between acquired and innate immunity (29).

1.2.4 T-Lymphocytes and cell-mediated immunity

In contrast to humoral immunity, cell-mediated immunity deals with intracellular pathogens, specifically viruses and some bacteria. Cell-mediated immunity is carried out by T-lymphocytes. T-lymphocytes use antigen-specific T-cell receptors (TCR) expressed on their surface to recognise antigens presented to them on the surface of an antigen presenting cell (APC), which ingest the pathogen proteins and digest them into small peptides. Thus, this type of acquired immunity is cell-mediated. The peptide fragments derived from the pathogen are transported to the surface of the APC and expressed there in conjunction with proteins termed major histocompatibility complex (MHC), after migration of the cells to the regional lymph nodes; in man MHC is termed human leukocyte antigen (33). 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 (from viruses and certain bacteria). MHC II binds peptides that originate from pathogens that have been phagocytosed by macrophages or endocytosed by APC (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 (34).

Activation of the TCR results in proliferation of T-lymphocytes, and also synthesis and secretion of the cytokine interleukin-2 (IL-2) from them. IL-2 has an autocrine activity promoting further proliferation and differentiation of T-lymphocytes. The expanded T-lymphocytes can migrate to sites of infection, injury or tissue damage. Cytotoxic T-lymphocytes ($CD8^+$) are stimulated by intracellular pathogens and they secrete cytotoxic enzymes which lyse the target cell, or secrete the antiviral cytokine interferon-γ (IFN-γ), or induce apoptosis of target cells (35). 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 (36).

The secretion of cytokines by antigen-activated $CD4^+$ T-lymphocytes causes recruitment of neutrophils and monocytes from the bloodstream to the site of infection, and also activation of monocytes. This type of cell-mediated immunity is termed delayed-type hypersensitivity (DTH), and is the primary defence against intracellular bacteria. The 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 (32).

Some aspects of the immune system may have undesirable consequences, such as host tissue damage by inflammatory mediators secreted by macrophages which are toxic not only to pathogens. Therefore, self-regulation of immune responses needs to be highly effective (37).

1.2.5 Cytokines

Cells of the immune system communicate either by direct cell to cell contact involving adhesion molecules or by producing chemicals which act as signals between cells. Cytokines are the most important chemical messengers produced. They are proteins which may have multiple activities on different cell types and they can regulate the activity of the cell that produced them or of other cells. Cytokines regulate the development, growth or activity of target cells by binding to specific receptors on their surface (38).

Some of the most important cytokines are tumour necrosis factor-α (TNF-α), IL-1, IL-6 and IL-8 produced by monocytes and macrophages. These cytokines are mediators for both the acquired and innate immune system, and thus they are a link between the two (39). Some of the actions of these cytokines are to activate monocytes, macrophages and neutrophils to eliminate infectious agents or tumour cells, to increase adhesion molecule expression on the surface of neutrophils and endothelial cells, to stimulate T- and B-lymphocyte proliferation, and to initiate the production of other proinflammatory cytokines. 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.

Inflammatory cytokines also mediate the systemic effects of inflammation, and their production is an important response to infection. However their overproduction has been shown to play an important role in some of the symptoms of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases and psoriasis (40).

1.2.6 Th1 and Th2 cell responses

Helper T-cells play an important role in the accumulation and activation of eosinophils and mast cells, and IgE production by B-cells. These characterise disorders which are associated with chronic inflammation. Thus, helper T-cells are believed to be important in the pathogenesis of allergic disease (41). Helper T-cells that encounter antigen for the first time produce mainly IL-2. These cells may differentiate into a Th0 cell population, which may further differentiate into helper T type 1 (Th1) or helper T type 2 (Th2) cells. These are the two broad categories that T-cells can be divided into and this is based on the different range of cytokines produced by them. Another, type of helper T-cells is the T-regulatory (T-reg) cells which produce IL-10 and play an important role in regulating the balance between Th1 and Th2. In addition, the differentiation of Th0 (naïve)

cells depends on cytokines. Specifically, the development of Th1 cells is mediated by IL-12 and IFN-γ, whereas the development of Th2 cells is mediated by IL-4 (42, 43).

As mentioned above these two types of cells have a specific profile of cytokine production and thus they induce different immune responses. Th1 cells produce IL-2 and IFN-γ. These activate macrophages, NK cells, cytotoxic T-lymphocytes and B-lymphocytes leading to cell-mediated immune responses against intracellular pathogens, and they are regarded as proinflammatory cytokines (44). Th1 activity is mostly induced by infection with bacteria, viruses and fungi. Inflammatory conditions, such as rheumatoid arthritis, psoriasis, and multiple sclerosis, are frequently associated with a bias towards Th1 cytokine responses (45).

Th2 cells produce IL-4, IL-5, IL-10 and IL-13. IL-4 stimulates IgE production by B-cells (antibody responses) and IL-5 activates eosinophils. IL-10, together with IL-4, suppresses cell-mediated immune responses by Th1 cells, and thus these cytokines may be regarded as anti-inflammatory (37). On the other hand, cytokines produced by Th1 cells, suppress Th2 cell activity. Th2 responses induce humoral immune responses, such as IgE-mediated activation of mast cells and baseophils to eliminate extracellular antigens (such as antigens of helminthic parasites). Biased responses towards Th2 have been associated with allergy development (43).

Cells with typical distinct Th1 and Th2 profiles have been demonstrated in mice. In humans, although some cells might have typical responses, there is no distinct division between the two types of cells. Helper T-cells in humans produce a mixture of Th1 and Th2 cytokines in various proportions (44). 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.

More recently, a novel helper T-cell subset which produces IL-17 has been identified as a distinct population from Th1 and Th2. These cells have been termed Th17 and they are the result of naïve T-cell differentiation though the effect of IL-1, IL-6, and IL-23. Th17 cells have been shown to be involved in the pathogenesis of infectious and autoimmune diseases leading to their exacerbation. Their existence has led to revision of the classic Th1/Th2 paradigm (46). There is evidence, although with discrepancies, that Th17 cells (as well as T-reg cells) may also contribute to the pathogenesis of some types of atopic diseases that have been considered to be Th2-mediated. Specifically, the observations on Th17 have provided with a new insight into the pathogenesis of ‘non-Th2-type’ asthma (i.e. ‘non-eosinophilic’/ ‘neutrophilic’ asthma) (41). Figure 1.3 represents Th1, Th2, Th17 and T-reg immune responses (41, 47).

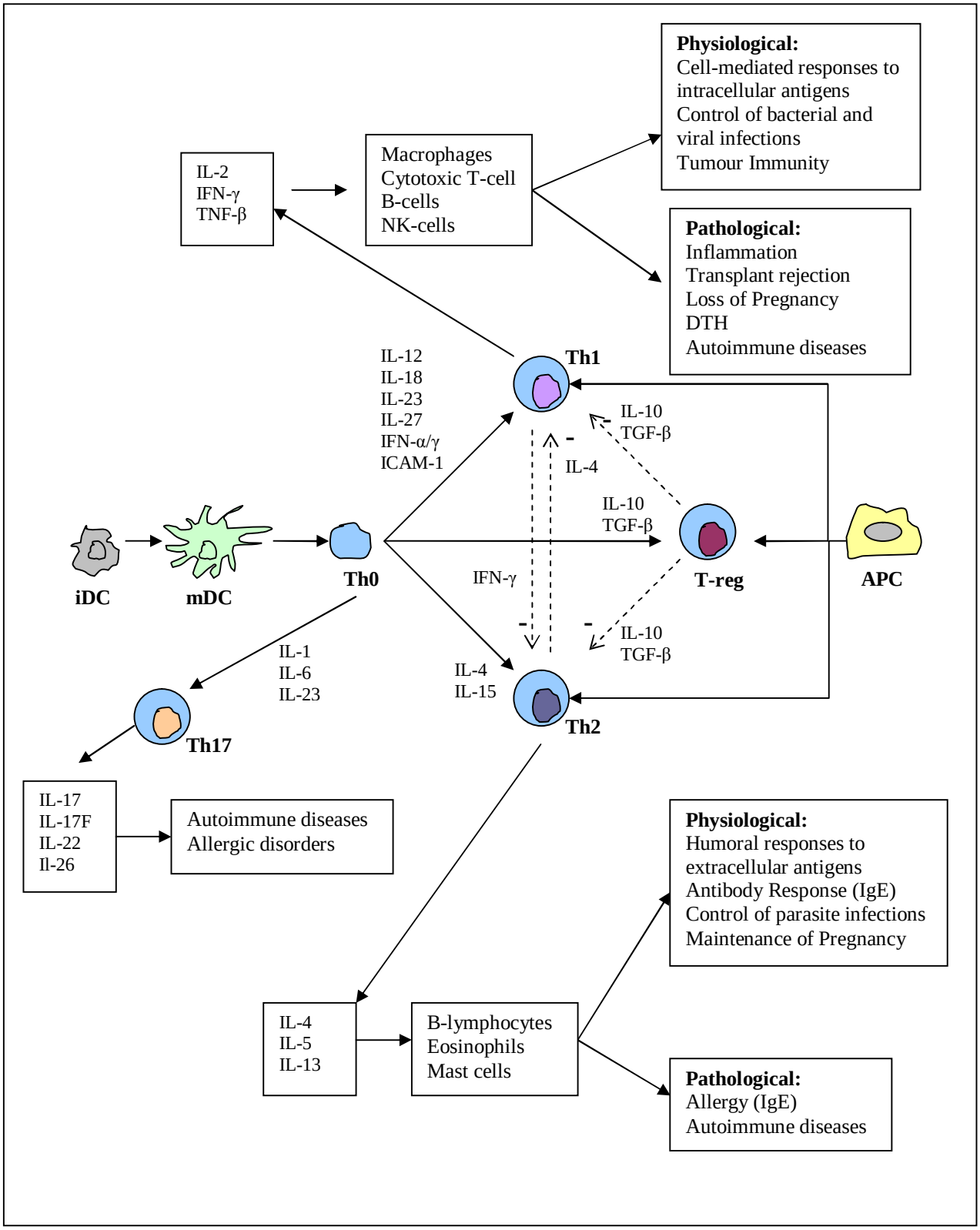


Figure 1.3 T-cell polarizing capacity of dendritic cells (DC), and the development and cytokine profiles of type 1 helper T-cells (Th1), type 2 helper T-cell (Th2), IL-17-producing helper T- cell (Th17) or T-regulatory cells (T-reg), and their roles in regulating physiological and pathological responses

iDC, immature DC; mDC, mature DC; Th0; naïve helper T-cell; ICAM, intercellular adhesion molecule; IL, interleukin; IFN, interferon; TGF; transforming growth factor; APC, antigen presenting cell; Ig, immunoglobulin; DTH, delayed type hypersensitivity.

Modified from (47) and (41)

1.2.7 Inflammation

Inflammation is part of the innate immune system response to infection or injury, and is characterised by redness, swelling, heat and pain. These are caused by increased blood flow and permeability across the blood capillaries, which allow large molecules (cytokines, complement, antibodies) and leukocytes to cross the endothelial wall and move to the point of infection or injury. Inflammation functions to eliminate invading pathogens and toxins, and to repair damaged tissue. The responses that occur during inflammation must be well controlled. The movement of cells into the infected site is induced by the up-regulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin on the surface of endothelial cells. These responses allow leukocyte binding and subsequent diapedesis (7).

The earliest cells to appear at inflamed sites are granulocytes, with monocytes, macrophages and lymphocytes appearing later. Activated macrophages and monocytes that arrive at the inflamed site form a range of inflammatory mediators such as cytokines, eicosanoids, nitric oxide, matrix metalloproteinases, reactive oxygen species, and kinins. These mediators are involved in pathogen destruction and communication between cells. They are also responsible for the systemic effects of inflammation on brain (fever, reduced appetite), on skeletal muscle (proteolysis), on adipose tissue (lipolysis), and on liver (acute phase protein synthesis). These inflammatory mediators provide the link between innate and acquired immune responses (5).

Uncontrolled inflammatory responses can cause damage to host tissues. Such inappropriate responses are characterised by hyperexpression of endothelial and leukocyte adhesion molecules, appearance of soluble forms of adhesion molecules in the circulation, appearance of leukocytes in unusual sites, and overproduction of inflammatory mediators.

1.2.8 The Immune system in health and disease: inappropriate immune responses

The functioning of the immune system is vital to maintain health and protect the body from pathogenic agents, injuries, and cancer cells. However, the immune system may in some individuals develop undesirable responses (48). One of these is the recognition and rejection of transplants which are being treated by the immune system as sources of foreign antigens. Another example is a dysfunction in the ability of the immune system to distinguish between 'self' and 'non-self', resulting in diseases such as rheumatoid arthritis, psoriasis and multiple sclerosis. These are autoimmune inflammatory conditions, since host tissues are being attacked, and they are characterised by chronic inflammation and a dysregulated Th1 response.

High concentrations of TNF-α, IL-1β, and IL-6 are implicated in some of the pathologic responses that occur in endotoxic shock, in acute respiratory distress syndrome, and in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. Chronic overproduction of TNF-α and IL-1 can cause adipose tissue and muscle wasting and loss of bone mass. These effects may account for alterations in body composition seen in cancer cachexia (40).

Normally, the immune system develops tolerance towards self-antigens, by destroying self-recognising T- and B-lymphocytes before maturation, or by a mechanism termed clonal anergy. These mechanisms can be disrupted because of a range of immunological abnormalities and genetic predisposition leading to autoimmune inflammatory diseases.

On the other hand, dysregulation of Th2 immune responses results in atopic diseases, including allergies, hay-fever, allergic rhinitis, eczema and asthma. In this case, the immune system is sensitised to usually harmless antigens from the environment, such as components of foods, pollens, house dust mite, animal fur, and these antigens are termed allergens. Inappropriate recognition of and/or responses to allergens includes IgE production by B-lymphocytes at initial exposure (40). The produced IgE binds to specific receptors on the surfaces of mast cells and basophils. This process is termed sensitisation. When the individual is re-exposed to the allergen, it will interact with the bound IgE, leading to cell activation and production of inflammatory mediators, especially histamine and the Th2 cytokines, IL-4, IL-5 and IL-10.

Finally, inflammation is now recognised to play a role in the pathology of other diseases, such as cardiovascular disease and neurodegenerative diseases of aging. Also, because of the fact that adipose tissue is a source of inflammatory cytokines, obesity,

metabolic syndrome, and Type 2 diabetes are believed to have an inflammatory component (7).

1.3 Early origins of atopic disease

1.3.1 Introduction

The aim of this section is to highlight that gene-environment interactions influence the development of atopic disease and that these interactions begin in early life. Health, nutrition and environmental exposures of the mother during pregnancy may influence the development of allergic diseases in the offspring.

The genetic component of allergic diseases is well established. Twin and family studies have shown the strong heritability of allergy (49-52). What is more, single nucleotide polymorphisms affecting Th2 pathways have been related to atopic disease (53-56). While, these polymorphisms contribute to the higher risk of developing allergy and allergic inflammation, their presence in the genome does not necessarily lead to development of allergic disease (57). Most atopic diseases have their origins in early life, their signs appear in early infancy and their progression is apparent all the way through childhood (58-62).

1.3.2 Foetal immune responses

Neonates are not immunologically naïve, and in fact immune responses are evident from early foetal life. Events that occur during foetal life contribute to the development of atopic disease. Neonates with higher risk of developing atopy appear to have different immune responses at birth from those with no family history of atopy and those who do not go on to develop atopic diseases (63).

Stem cells can be found in the human yolk sac at 21 days. The first lymphocytes are found in the thymus by the week 9 of gestation. Lymphocytes are also found in different organs like the lungs and the gut (14 weeks gestation). B-cells are present in circulation by 16 weeks gestation. What is more, the complete process of immune sensitisation is known to occur in the neonate, and antigen-specific responses at birth are not unusual. Antibody responses (IgG, IgM) can be detected in neonates after antigen exposure of the mother or of the foetus during pregnancy. Also, it has been shown that circulating blood mononuclear cells can specifically proliferate as a response to allergens from 22 weeks gestation (64).

Specific responses of the foetus to common environmental factors increase with progression of the pregnancy, and at birth most neonates are able to mount such responses. It has been shown that strong proliferative specific responses to an allergen at birth are

associated with a higher risk of developing inappropriate immune responses to that allergen later on in childhood (64).

1.3.3 Immune responses in pregnancy

Maternal cell-mediated immune responses to foetal and paternal antigens are suppressed during the course of pregnancy, and this allows the normal progression of pregnancy. The balance of the maternal immune response is shifted away from cell-mediated immunity to humoral immunity, which is less damaging. This shift in balance of maternal immunity is a result of cytokines produced from tissues in the foeto-placental unit, which are characteristic of Th2 responses (64). The Th2 cytokines produced in normal pregnancy may also lead to biased suppression of Th1 responses. Thus, immunologic changes during pregnancy lead to polarization towards Th2 and T-regulatory responses (64, 65).

Cytokines produced by decidual tissues include IL-1α, IL-1β, IL-6, IL-13, IL-4, and IL-10. The production of these Th2 and regulatory cytokines is regulated by the interaction between the mother, the placenta and the foetus. Also, the cytokines produced modulate foetal immunity so that the immune responses detected in neonates are biased towards the Th2 phenotype, so that the immune system of the foetus is polarised towards the same direction (Th2/T-regulatory) as in the pregnant woman (66). This is balanced by the rapid postnatal maturation of Th1 responses. Thus, although immune system maturation begins *in utero*, it continues during the first two years of life with appropriate antigen exposure.

Th2 and T-regulatory cytokines can be detected in the amniotic fluid during the second trimester of pregnancy. As the foetus swallows amniotic fluid, these cytokines will be swallowed by the foetus. Also, there is some aspiration of the amniotic fluid, through the permeable skin of the foetus, into mucosal surfaces of the respiratory tract during foetal respiratory movements (63).

The immune system of the foetus is not fully functional and its gastrointestinal tract is sterile. The gut becomes colonised after exposure to bacteria during birth and postnatally from the mother's skin, immunologic factors in breast milk, and antigens in formulas and weaning foods. Furthermore, it has been shown that immune active cells (macrophages, dendritic cells, T- and B-lymphocytes) are present in the foetal gut from early gestation. Maturation of these cells in the foetal gut possibly results from exposure to cytokines as well as food and inhalant allergens in the amniotic fluid (67).

Antibodies of maternal origin (IgG and IgE) can be detected in the amniotic fluid from early gestation. Since receptors for these antibodies exist in the foetal gut, it is possible that their presence in the amniotic fluid facilitates the uptake of antigens by APC, and thus promotes sensitisation to allergens present in the amniotic fluid even in low concentrations (68). Therefore, the formation of IgE and IgA starts *in utero* and leads to the development of specific T-cell responses against environmental and food antigens particularly during the third trimester of pregnancy. Antigen-reactive T-cells can be detected in the cord blood of almost all infants supporting the idea of early sensitisation (69).

The foetus can also be exposed to antigens via its circulation during the third trimester of pregnancy. IgG linked to antigens and allergens are actively transported across the placenta into the foetal blood stream. Higher exposure to allergens during pregnancy leads to higher production of allergen-specific IgG antibodies in the mother. Higher IgG levels to specific allergens are associated with lower risk of sensitisation to these allergens in the offspring. The relationship between the risk of allergic sensitization in the offspring and maternal exposure during pregnancy is a bell shaped curve (70). Thus, reducing exposure to allergens, such as house dust mite, during pregnancy might have adverse effects increasing the risk of sensitisation to these specific allergens. On the contrary, high levels of exposure to allergens during pregnancy may be protective for the offspring.

Consequently, the concentration of the antigen to which the foetus is exposed during pregnancy, and the timing, the nature (bound to IgG or free), and the route of exposure affect the development or not of sensitization and clinical outcomes of atopy (64).

1.3.4 Early life nutrition and development of atopic disease

Epidemiological data show that the incidence of diseases with an immunological basis has increased dramatically over the past three to four decades especially in westernised environments (71, 72). For example, the incidence of atopic diseases has increased, particularly among children (73, 74). This increased incidence has been related to environmental changes that have occurred during the past years. Leibowitz *et al.* (75) suggested that high levels of sanitisation at home during childhood are associated with higher risk of multiple sclerosis. Strachan (76) observed that the order of birth and size of the family were inversely associated with the risk of allergic rhinitis. This finding led to the hygiene hypothesis, according to which infections within households in early childhood have a protective role against allergic rhinitis (76). Bach (77, 78) has reviewed the evidence and mechanisms supporting that the main factor of increased prevalence of allergic and autoimmune diseases in industrialised countries is the decreased incidence of

infectious diseases over the past 30-40 years. Figure 1.4 shows that the incidence of infectious diseases has decreased and this may be related to the increase in immune disorders (figure from (77)).

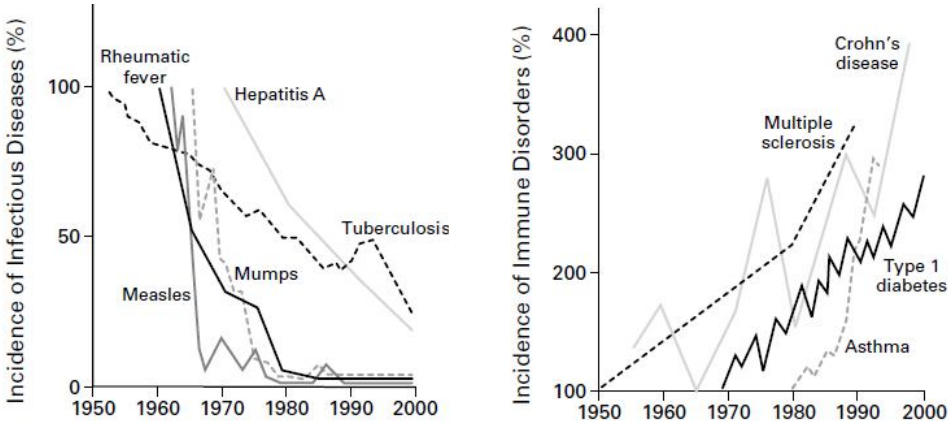


Figure 1.4 Inverse relation between the incidence of infectious diseases and immune system disorders from 1950 to 2000
Figure from (77)

However, some of the most important environmental changes that have occurred over the last decades are dietary. Changes in diet that have occurred during the last 30-40 years that have been related to increased sensitisation of the immune system to allergens include the increased intake of n-6 PUFA followed by a subsequent decrease in n-3 PUFA. Specifically, Black & Sharp (79) and Hodge *et al.* (80) were the first to argue that the increased incidence and prevalence of childhood allergic disease has coincided with the increased intake of n-6 PUFA (linoleic acid). Also, the lower intake of antioxidant vitamins and minerals, and changes in diet that have lead to an altered pattern of the gut microflora have been associated with the increased rates of atopic disease (47, 81).

Maternal intake of fruit and vegetables (antioxidant intake), and fish (n-3 PUFA intake) during pregnancy will determine maternal antioxidant and n-3 PUFA status, which may subsequently determine the status of these nutrients in the neonate. It has been shown that n-3 PUFA status in maternal blood and breast milk is lower in mothers of children who go on to develop atopy (82-88). Also, it has been shown that cord blood levels of EPA and DHA are lower and ARA is higher in infants who develop atopy later on in life and also in infants of atopic mothers (89-91). Additionally, low cord selenium and iron have been associated with the development of atopic diseases in the offspring (92). Moreover, atopic infants have been shown to have a different gut microflora compared to non-atopic infants (47, 81). Thus maternal n-3 PUFA and antioxidant status, as well as maternal gut

microflora, may be important in determining the development of atopy in the offspring. This underlines the importance of the early life environment and maternal diet during pregnancy in the development of atopic disease (81).

Prescott and Dunstan (93) have recently reviewed the effects of dietary LC PUFAs on early immune development and their potential role in immune disease development or prevention. The potential effects of LC-PUFA may be of great importance during early life. Potential pathways through which LC-PUFA could influence immune programming include effects on: local tissue factors (production of lipid-derived inflammatory mediators, oxidative stress), neonatal APC function, T-regulatory cell function, T-cell function, B-cell function (antibody production), and end-organ factors (affecting clinical phenotype). The review concluded that the relevance of these effects to the prevention of disease is not clear, and that intervention studies during pregnancy are needed in order to address more definitely the potential beneficial effects of early LC-PUFA exposure on allergy prevention in the offspring (93).

In conclusion, nutrition in early life may affect immune development by providing: antigens to which the immune system will acquire tolerance, nutrients which can modulate immune responses (such as n-3 PUFA and antioxidants), and factors that influence the intestinal flora (such as dietary fibre, prebiotics and probiotics) (47).

1.4 Fatty Acids and the Immune system

This part of the introduction aims to present the possible mechanisms by which fatty acids may affect immune responses.

There are many studies of fatty acids and the immune system. These have been reviewed elsewhere (2, 5, 7, 94, 95). The clearest mechanism by which fatty acids can influence the immune system is by altering eicosanoid patterns and this will be described in sections 1.4.1-1.4.3. The present PhD thesis examined T cell responses in the blood of pregnant women and whether these are modulated by consumption of salmon. As the pattern of production of Th1 and Th2 cytokines has been measured in the current thesis, an overview of the literature on this topic will be provided (section 1.4.4).

1.4.1 Eicosanoids

The link between fatty acids and the immune system relates to the composition of immune cell membrane phospholipids. The fatty acid composition of cell membrane phospholipids can influence various membrane activities, which can influence cellular responses. For example, the fluidity of the membrane is regulated in part by the fatty acid composition of its constituent phospholipids. Membrane fluidity is important for the activity of membrane proteins such as receptors, transporters and enzymes. The responsiveness of immune cells to a stimulus can be altered by membrane fluidity. What is more, the production of intracellular signals, such as diacylglycerol (DAG), inositol phosphates, and ceramide, from membrane phospholipids, is dependent on the activity of phospholipase enzymes which can be altered by the fatty acid composition of the substrate phospholipids. Another group of mediators, the eicosanoids, are generated from PUFA released from cell membrane phospholipids. Consequently, the fatty acid composition of immune cells is important for the regulation of the responses and functions of these cells.

Eicosanoids, as indicated by their name, are a group of mediators whose precursors are 20-carbon atom PUFA (ARA, 20:4n-6; EPA, 20:5n-3). These PUFA are contained in the phospholipid bilayer of cell membranes, with ARA being contained in most cell membranes in relatively large amounts. Eicosanoid synthesis involves PUFA mobilisation from the cell membrane by various phospholipase enzymes, most notably phospholipase A2. The free PUFA is converted by cyclooxygenase enzymes (COX) into prostaglandins (PG), thromboxanes (TX) and related compounds, or by lipoxygenase enzymes (LOX) into

leukotrienes (LT) and related compounds. Figure 1.5 presents the synthesis of eicosanoids from PUFA (7).

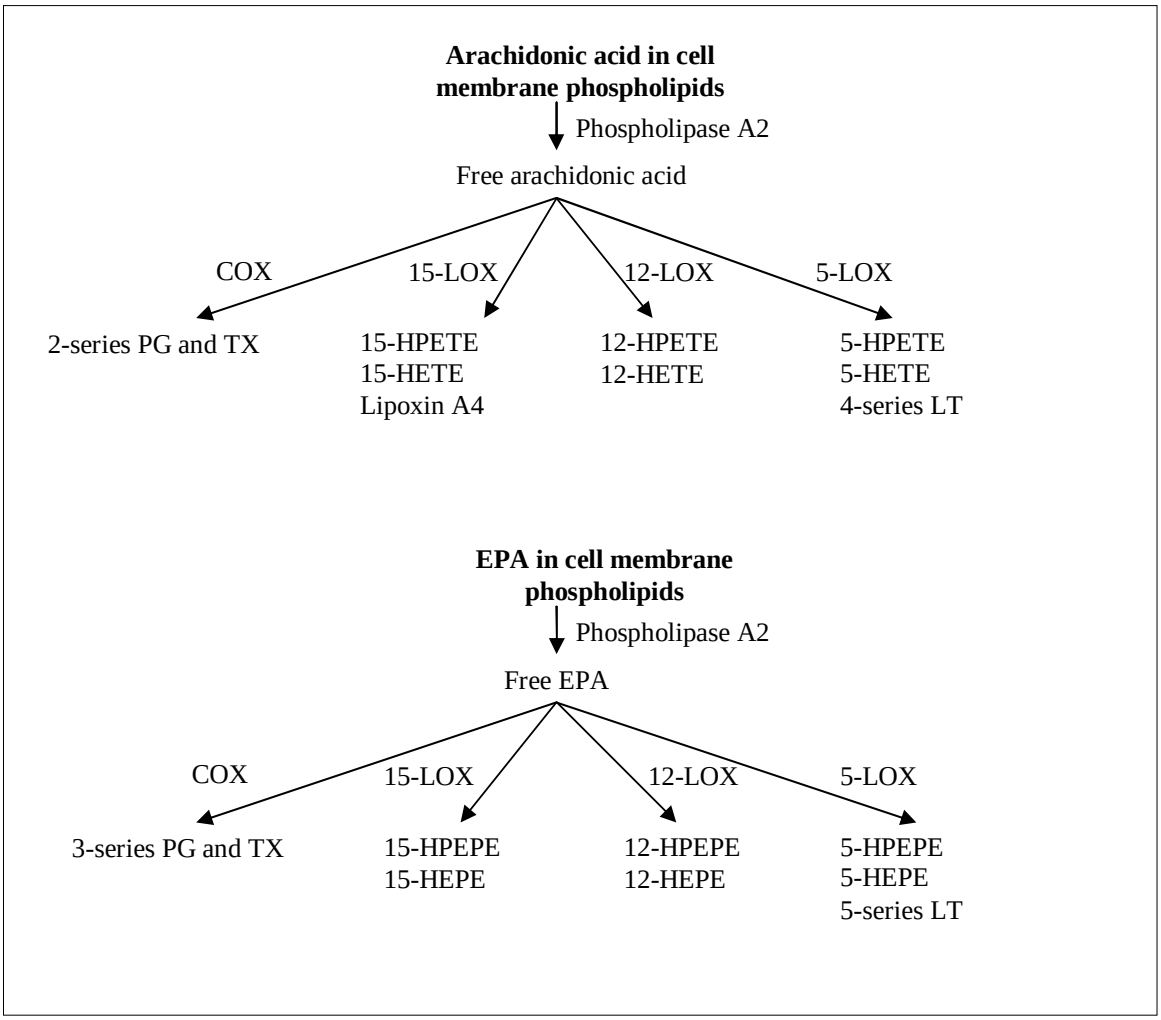


Figure 1.5 Synthesis of eicosanoids from arachidonic acid and eicosapentaenoic acid (EPA)
COX, cyclooxygenase; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPEPE, hydroperoxyeicosapentaenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane.
Adopted from (7)

ARA is the principal precursor of eicosanoids. 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 (96, 97). The effects of eicosanoids may vary even if these have the same precursor, and in addition, a single eicosanoid may have opposing effects on different cell types. 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 sometimes 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, inhibition of IFN-γ, TNF-α and IL-1 production). Recent studies have shown that PGE_2 induces the production of IL-6 by macrophages (98), induces COX-2 in fibroblast cells and subsequently stimulates its own production (98), inhibits 5-LOX leading to decreased production of 4-series LT (99), and induces 15-LOX promoting the formation of lipoxins (99, 100), which have been shown to have anti-inflammatory effects (101, 102). LTB_4 also has proinflammatory effects such as increase of vascular permeability and enhancing the production of inflammatory cytokines, but also inhibits lymphocyte proliferation. Table 1.4 lists the anti- and pro-inflammatory effects of two different eicosanoids (7).

Table 1.4 Pro- and anti-inflammatory effects of prostaglandin E_2 and leukotriene B_4

Eicosanoid	Pro-inflammatory effects	Anti-inflammatory effects
PGE_2		
	Induces fever	Inhibits Th1-type response
	Increases vascular permeability	Inhibits production of TNF-α and IL-1β
	Increases vasodilatation	Inhibits T-cell proliferation
	Enhances pain	Inhibits 5- and 15-LOX
	Promotes Th2-type response	
	Promotes IgE production by B-cells	
LTB_4		
	Increases of vascular permeability	
	Enhances of local blood flow	
	Chemotactic agent for leukocytes	
	Induces release of lysosomal enzymes	
	Induces of release of reactive oxygen species by granulocytes	
	Increases production of TNF-α, IL-1β	
	Promotes IgE production by B-cells	

LOX, lipoxygenase; LT, leukotriene; IL, interleukin; TNF, tumour necrosis factor; Ig, immunoglobulin; Th1, helper T-cell type 1; Th2, helper T-cell type 2
Adapted from (7, 103)

1.4.2 Arachidonic acid and inflammatory mediators

The amount of ARA in immune cells can be increased by including ARA in the diet of rats (104), or by increasing its intake from the diet in humans (105). 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 (7, 106).

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 (104). 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 endotoxin stimulated mononuclear cells (107). However, it was shown that the production of inflammatory cytokines (TNF-α, IL-1β, IL-6) by these cells was not affected (107, 108). Furthermore, T-cell responses, the generation of reactive oxygen species (superoxide) by neutrophils and monocytes (108), and the concentrations of adhesion molecules (VCAM-1, ICAM-1, E-selectin) in plasma were not affected (108).

1.4.3 LC n-3 PUFA and eicosanoid production

Increased dietary intake of EPA and DHA results in increased proportions of these fatty acids in immune cell membranes phospholipids (106, 109, 110). The incorporation of long-chain n-3 PUFA occurs in a dose-response fashion and is accompanied by decreased levels of ARA in immune cell membranes (110). Consequently, there is less substrate available for the synthesis of ARA-derived eicosanoids. In accordance to this, fish oil supplementation of the human diet has been shown to result in decreased production of PGE_2 (111, 112), TXB_2 (113), LTB_4 (109, 114), 5-hydroxyeicosatetraenoic acid (5-HETE) (109, 114), and LTE_4 (115) by inflammatory cells.

Furthermore, EPA can be converted to 3-series PG and TX, and 5-series LT, by COX and 5-LOX respectively. 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 (109, 114, 115), although it was more difficult to demonstrate the production of PGE_3 (116). 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.

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 appear to have anti-inflammatory actions. (117-119). 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 (95, 120).

1.4.4 Effects of fish oils on T-cell function

The effects of dietary fish oils cannot be predicted based on the EPA and DHA-derived eicosanoids produced. As mentioned above, dietary n-3 PUFA decrease production of ARA-derived eicosanoids (PGE_2), and they give rise to eicosanoids and eicosanoid-like mediators (E- and D-resolvins) with varying actions. Because of the effects of eicosanoids on Th1 and Th2 responses and lymphocyte proliferation, it could be speculated that fish oils could influence T-cell functions through the production of eicosanoids. However, because fish oils may affect the production of a mixture of anti- and proinflammatory mediators, it is not possible to predict their precise effect on immunomodulation. The influence of fish oils on T-cell functions and Th1/Th2 cytokine production have been reviewed in detail elsewhere (1, 47, 121). The results of the most significant in vitro, animal feeding and human supplementation studies in this area are summarised in this section.

It has to be mentioned at this point that most human studies used blood lymphocytes as a mixture with monocytes. These preparations are described as peripheral blood mononuclear cells (PBMC) and they contain typically 85-90% lymphocytes and 10-15% monocytes (121). Proliferation in response to mitogens is the most widely used test of lymphocyte function. Mitogens used to stimulate T-lymphocyte proliferation are concanavalin A (Con A) and phytohaemagglutinin (PHA). Proliferative response is determined either by measuring the increase in number of cells or by measuring the incorporation of [^{3}H]thymidine into the DNA.

In vitro studies have shown that adding EPA and DHA in culture inhibits the production of IL-2 and proliferation of lymphocytes (1, 121). In the study of Soyland *et al.* EPA and DHA exerted a strong dose-dependent inhibitory effect on proliferation of

mitogen- or antigen-stimulated human T-cells *in vitro*. COX and LOX inhibitors did not affect the anti-proliferative effect of EPA (122). In the study of Khalfoun *et al.* (123, 124) it was shown that EPA and DHA had no effect on PHA-induced lymphocyte agglutination, but they strongly inhibited the lymphocyte proliferative response to PHA. This inhibition was dose-dependent and more potent with DHA than EPA. However, EPA and DHA did not inhibit IL-2 stimulated lymphocyte proliferation (124). Similarly, in another *in vitro* study on human lymphocytes, it was shown that the proliferative response to mitogens (PHA, Con A) was inhibited in a dose-dependent manner (125). Furthermore, the inhibition of T-cell proliferation was associated with decreased production of IL-2, TNF-α, and IFN-γ. Calder *et al.* (126) showed that PGE_2 concentrations in the medium taken from lymphocytes cultured in the presence of EPA and DHA were not associated with the inhibitory effects of these fatty acids on lymphocyte proliferation. Also, PGE_3 did not inhibit lymphocyte proliferation, except at high concentrations. Lymphocyte proliferation was not affected by inclusion of inhibitors of phospholipase A2, COX or LOX in the cultures. Thus, inhibition of lymphocyte proliferation by n-3 PUFA appears to be independent of eicosanoid production (122, 126). The results of these studies show that *in vitro* n-3 PUFA influence T-cell function by inhibiting proliferation and IL-2 production but that the effects probably do not involve a change in eicosanoid profiles.

The results of animal feeding studies showed high levels dietary fish oil decreased lymphocyte proliferation (127, 128), and resulted in lower production of IL-2 and IFN-γ (128, 129). In the study of Jolly *et al.* (128) murine T-cell function was examined in response to a low fat, short term (10 days) diet enriched with EPA or DHA. It was shown that Con A-induced T-cell proliferation in splenocyte cultures was suppressed by dietary EPA and DHA, and this suppression appeared to be mediated by a reduction in IL-2 secretion. This study showed that even at lower levels of intake, both EPA and DHA inhibit lymphocyte proliferation and IL-2 production (128). The study of Wallace *et al.* (129) compared the effects of different types of oils (coconut, safflower, fish oil) with a low fat diet fed to mice for 6 weeks. IFN-γ production was decreased by safflower or fish oil feeding. IL-4 production was lowest by lymphocytes from fish-oil fed mice, although it was not significantly affected by diet. The ratio of Th1/Th2 cytokine production (IFN-γ/IL-4) was lower from lymphocytes of mice fed safflower or fish oil diets. Also, IL-2, IFN-γ, and IL-4 mRNA levels were lowest in cells from mice in the fish oil diet group. The ratio of IFN-γ mRNA/IL-4 mRNA was lowest in cells of mice fed fish oil. *In vitro* results showed that the n-3 PUFA found in fish oil were the most potent inhibitors of IL-2 production. It was concluded that n-3 PUFA inhibit production of Th1-type cytokines with

little effect on Th2 type cytokines. In the study of Sanderson *et al.* (130) rats were fed for 12 weeks with different high fat diets (coconut, olive, safflower, evening primrose, or fish oil) or a low fat diet. It was shown that after feeding with fish oil mitogen-stimulated spleen lymphocyte proliferation was inhibited (130, 131). Therefore, the results of animal feeding studies show that fish oil decreases lymphocyte proliferation and skews T-cell responses away from the Th1-type response (121).

Human supplementation studies with EPA and DHA have shown differential effects on lymphocyte functions. Meydani *et al.* (111) showed that supplementation of healthy volunteers with fish oil providing 2.4 g EPA plus DHA/day for three months resulted in decreased PHA-stimulated proliferation of lymphocytes and also decreased IL-2 production in women aged 51-68 years (but not in younger women aged 23-33 years). In another study of Meydani *et al.* (132) it was shown that supplementation of a low fat diet with 1.2 g EPA plus DHA/day decreased T-cell proliferation in subjects older than 40 years old. Another study provided 2 or 4 g EPA plus DHA/day, and this resulted in decreased PHA-stimulated PBMC proliferation in healthy volunteers and in newly diagnosed Type 1 diabetic patients (133). Moreover, Gallai *et al* (134) showed that 5.2 g/day EPA plus DHA decreased IL-2 and IFN-γ production by stimulated PBMC. In the study of Thies *et al.* (105) 1 g EPA plus DHA/day (720 mg EPA plus 280 mg DHA) for 12 weeks decreased lymphocyte proliferation, but did not affect IL-2 or IFN-γ production in healthy subjects aged 55-75 years. In the study of Endres *et al.* (135) it was shown that supplementation with EPA and DHA resulted in suppressed synthesis of IL-2 from stimulated PBMC 10 weeks after the end of supplementation, and also suppressed PHA-induced proliferation of PBMC. However, the effect of n-3 PUFA on IL-2 production was no longer evident at the end of the study (135). Consequently, several studies have demonstrated that dietary n-3 PUFA supplementation in humans significantly reduces lymphocyte proliferation to mitogens.

On the contrary, there are a number of studies that showed no effects of human supplementation with n-3 PUFA. Specifically, it was reported that supplementation of healthy subjects with 3.2 g EPA plus DHA/day for 12 weeks did not alter lymphocyte proliferation and IL-2 and IFN-γ production (106). However, the authors stated that the lack of effects might have been associated with the level of α-tocopherol in the supplement. One study has reported that moderate dose of fish oil supplementation (0.3, 1 and 2 g EPA plus DHA/day) for 4 consecutive weeks each (total of 12 weeks) increased T-cell proliferation and IFN-γ production (112).

Finally, there are a few studies investigating the effects of DHA alone. The study of Kelley *et al.* (107, 136) found no effect of supplementation of men aged 20-40 years with 6 g DHA/day for 90 days on lymphocyte proliferation or the number of T-cells producing IL-2. What is more, another study showed that supplementation with 720 mg DHA/day did not affect lymphocyte proliferation or IL-2 and IFN-γ production in healthy subjects aged 55-75 years (105, 108). These studies show that either high or low levels of DHA supplementation alone do not affect T-cell proliferation and cytokine production.

In conclusion, both *in vitro* and animal feeding studies showed that n-3 PUFA inhibit T-cell proliferation and production of IL-2 and IFN-γ. Human supplementation studies indicate that high levels of fish oil supplementation may inhibit T-cell responses. However, lower levels of supplementation do not exhibit such effects. Studies that have used levels of supplementation between the previous two levels had conflicting results. Inconsistency in results may be related to differences in subject characteristics (age, gender, health status, background diet), study design (dose and duration of supplement), as well as to differences in experimental methods (cell preparation, cell culture, cytokine assays) (121). All in all, the evidence available shows that fish oils may affect T-cell responses and function independently of eicosanoid production. Figure 1.6 summarises the anti-inflammatory effects of LC n-3 PUFA discussed in this part.

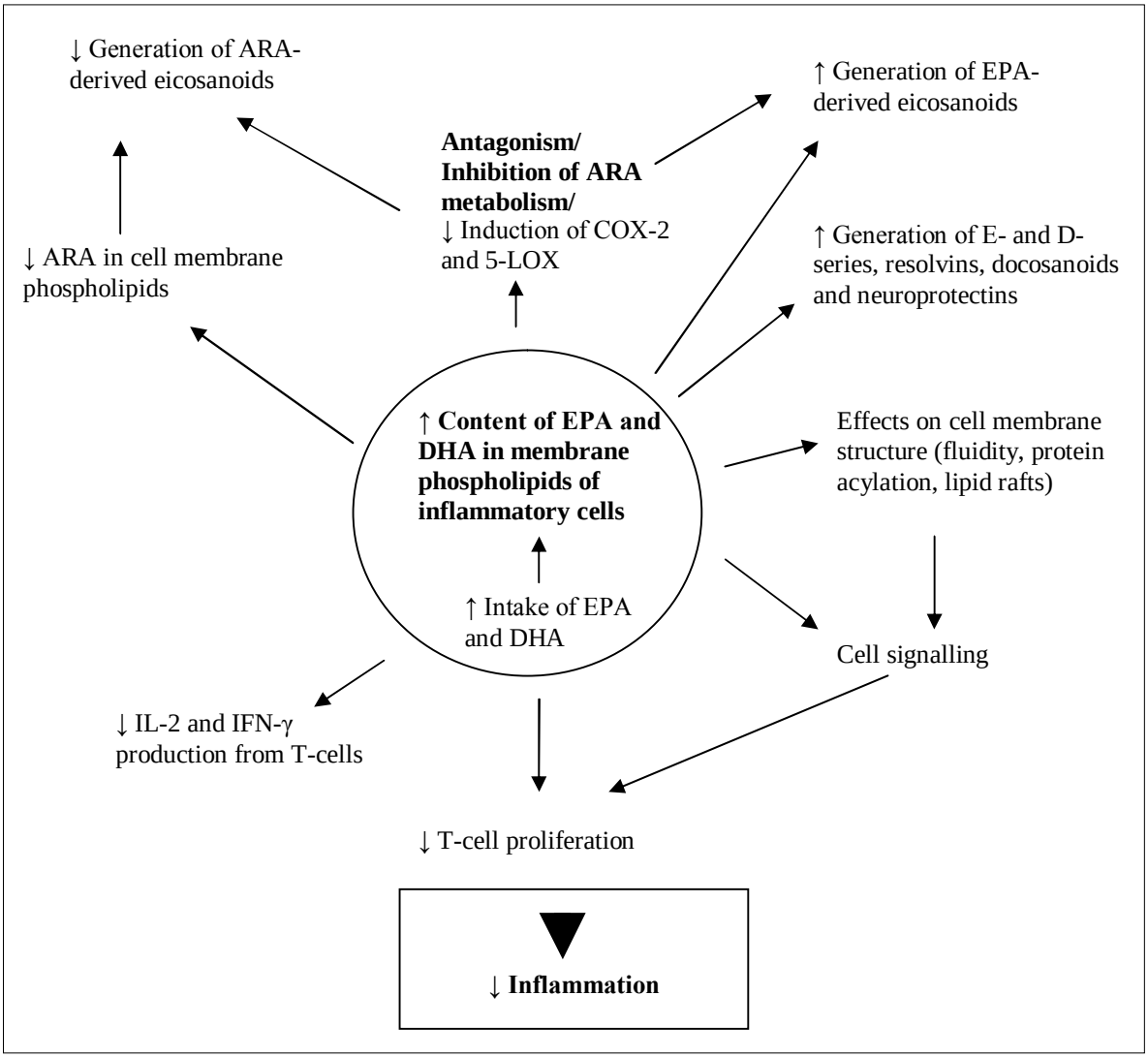


Figure 1.6 Anti-inflammatory effects of long-chain n-3 polyunsaturated fatty acids
AA; arachidonic acid; COX, cyclooxygenase; LOX, lipoxygenase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; IL, interleukin; IFN-γ, interferon-γ

1.4.5 Fish oil and chronic inflammatory diseases

1.4.5.1 Th1 responses

Chronic inflammatory conditions that are related to Th1-type response dysregulation are characterised by inappropriate production of ARA-derived eicosanoids (PGE_2 and LTB_4) and inflammatory cytokines. Since n-3 PUFA from fish oil act to decrease ARA-derived eicosanoid production, it has been suggested that fish oils may have a preventive or therapeutic role for these diseases. Supplementation trials have been conducted for most of these diseases. Clinical trials provide good evidence of the anti-inflammatory effects of fish oils on rheumatoid arthritis (137-139) and inflammatory bowel diseases (Crohn's disease and ulcerative colitis) (140, 141), and are associated with clinical improvements. However, the therapeutic effect of fish oils on other conditions, such as multiple sclerosis and systemic lupus erythematosus, is not clearly evident (142-144).

1.4.5.2 Th2 responses: fish oil and atopic disease

Atopic diseases, which are also associated with dysregulation of the Th2-type response, are associated with production of ARA-derived eicosanoids such as PGD_2, LTC_4, LTD_4 and LTE_4 (produced by mast cells to mediate pulmonary inflammation in asthma). What is more, PGE_2 regulates the activities of macrophages and lymphocytes, inhibits the production of Th1-type cytokines (IFN-γ, IL-2) without affecting the production of Th2-type cytokines (IL-4, IL-5), regulates T-cell lymphocyte differentiation promoting the development of the Th2 phenotype (145), and stimulates B-cells to produce IgE (146). Thus, ARA-derived eicosanoids have been suggested to play an important role in the aetiology of asthma, and in addition, PGE_2 may be involved in the development of allergic disease. The hypothesis that has evolved is that an increased intake of n-6 PUFA accompanied by a low intake of n-3 PUFA has played a causal role in increased asthma incidence in the last 30 to 40 years (79, 80).

A number of studies have been conducted investigating the possible therapeutic affects of fish oils on asthma, after its development. It has been reported that fish oil supplementation in patients with asthma has anti-inflammatory effects, such as decreased 4-series LT production (147-149) and leukocyte chemotaxis (148, 149). Uncontrolled and open-label trials of fish oil have also shown clinical benefit of fish oil on asthma (150). Schachter *et al.* (150) and Woods *et al.* (151) have reviewed randomised controlled and other type of supplementation trials of fish oil in asthmatic patients. Woods *et al.* (151) concluded that there was no consistent effect on forced expiratory volume in one second or

peak flow rate, both measures of lung function, or on asthma symptoms, asthma medication use or bronchia hyper-reactivity. Similarly, in the review conducted by Schachter *et al.* (150), it was concluded that the efficacy of n-3 PUFA supplementation as a treatment for asthma in adults and children is not clearly evident. However, there are some studies which show that there may be subgroups of asthmatic subjects who benefit greatly and others who do not benefit from long-chain n-3 PUFA (151, 152). Further studies are needed to be conducted in this area in order to provide strong evidence of the potent therapeutic effects of fish oils on asthma.

There are biochemical indicators showing that there is an inverse relationship between n-3 status and atopic disease. A study showed that EPA, docosapentaenoic acid, and DHA proportions were higher in the umbilical cord serum phospholipids from non-allergic compared to allergic mothers (91). Higher n-3 PUFA in breast milk were associated with a decreased likelihood of atopy in the infants (82). Non-atopic children between 12 to 15 years old had higher proportion of DHA and of total n-3 PUFA in serum phospholipids compared to children with asthma and/ or atopic dermatitis (153). It is now recognised that sensitisation to allergens occurs early in life (69).Thus, maternal diet may be very important in determining predisposition to atopy. Studies investigating early exposure to long-chain n-3 PUFA show in overall modulation of the development of atopic diseases. These studies are reviewed in Section 1.5.

1.5 Atopy risk in infants and children in relation to early exposure to fish, oily fish, or LC n-3 PUFA: A systematic review

This review collates the evidence available on the effects of fish intake and fish oil supplementation of pregnant or lactating mothers on immune and atopy outcomes in their offspring, as well as the effects of fish intake and fish oil supplementation during infancy or childhood on immune and atopy outcomes on those infants/children. This review has been recently published by Kremmyda L.S., Vlachava M., Noakes P.S., Diaper N.D., Miles E.A., and Calder P.C. in Clinical Reviews in Allergy and Immunology (103).

1.5.1 Epidemiological studies relating early fish exposure to atopy outcomes in infancy or childhood

The aim of this section is to describe and interpret epidemiological studies on the effects of maternal fish intake during pregnancy or lactation on atopic/allergic outcomes in the offspring of those mothers, as well as the effects of fish intake during infancy/childhood on atopic/ allergic outcomes in those infants/children.

1.5.1.1 Studies investigating the effect of maternal fish intake during perinatal life on atopic outcomes in infants or children

Table 1.5 includes all studies published to the present 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 (1950-2009) and Embase (1980-2009) 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.

Studies investigating the effect of maternal fish intake during pregnancy on atopic outcomes in infants or children

Study design

Out of the five studies which examined the effect of maternal fish intake during pregnancy three studies were prospective cohort (154-156), one study was case-control (157), and one study was retrospective cohort (158). All five studies had large sample

sizes, with the largest being in the studies of Willers *et al.* (156) and Sausenthaler *et al.* (154). The age range of children taking part in these studies was between 2-16 years. Children who took part in the three prospective cohort studies were followed-up for 6, 2 and 5 years (154-156) for each study respectively.

Table 1.5 presents only statistically significant results referring to maternal fish intake in relation to atopic or allergic outcomes in infants or children. Other results reported by these studies that were either not significant or not related to fish intake are not included here.

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) (155, 157, 158) and two used a semi-quantitative FFQ (154, 156). FFQ varied in frequency categories and also in time point of administration [during pregnancy (156), shortly after birth (154, 155) or retrospectively a long time after birth (157, 158)] and way of administration [self-administered (156) or interviewer-administered (154, 155, 157)].

Two of the studies examined the effect of total fish consumption during pregnancy on childhood/infancy atopic outcomes (154, 157, 158). Three studies examined the effect of different fish categories (155-157), and two of them found statistically significant results for oily fish (156, 157). It has to be pointed out that, although Romieu *et al.* (155) found a statistically significant protective effect 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. Regarding fish consumption frequency, Salam *et al.* (157) observed statistically significant results when comparing the effects of 'monthly intake' versus 'never' whereas the other four studies (154-156, 158) observed statistically significant results when comparing the effects of different weekly consumption frequencies. Analysing different types of fish and consumption frequencies could possibly explain the heterogeneity in the extent of the protective effect between the studies.

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 (154, 155). 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 (156, 158).

Moreover, the definition of 'oily fish' was not consistent if it existed, which gives rise to the issue of dietary exposure misclassification. For example, Salam *et al.* (157) 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.* (155) and Willers *et al.* (156).

Generally, FFQ is an effective method of assessing nutrient intakes during pregnancy in observational studies of fairly large sample sizes. However, a complete picture of the rest of the diet is needed in order to identify other food categories/items related to the outcome measure or that could be possible confounding factors. In this way, apart from fish categories it is important other major food categories/food items to be considered so as to control for other components of the diet, such as total energy intake, dietary fat and antioxidant intake. A FFQ reflecting total and oily fish intake throughout pregnancy and which is not administered retrospectively after delivery (in order to reduce recall bias) nor is self-administered would be ideal.

Confounding factors

All of the studies adjusted for most recognized confounding factors. Romieu *et al.* (155) included type of fish as a confounding factor and Sausenthaler *et al.* (154) adjusted for all dietary variables included in their FFQ. Three of the studies controlled for breastfeeding (154, 156, 157). All five studies controlled for maternal atopy/asthma and all, with the exception of Salam *et al.* (157), controlled for maternal smoking. It should be mentioned that, because fish consumption may be a marker of specific lifestyle, it is important for the studies to adjust for socio-economic factors such as social class and parental education, as well as smoking. All of the studies in Table 1.5 have adjusted for most of these confounding factors.

Outcome measures

There was great heterogeneity among the five studies with regard to outcome measures and their assessment. Salam *et al.* (157) focused on asthma, Calvani *et al.* (158) focused on allergic sensitisations, while Sausenthaler *et al.* (154), Romieu *et al.* (155), Willers *et al.* (156) included various outcome measures such as eczema, atopic wheeze, allergic sensitisation, or hay-fever. Also, each of the studies assessing clinical outcomes used a parental questionnaire which most of the times 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 mild or at early stages (157).

Results

There is consistency between the findings of these five studies since each 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.* (157) the association between maternal oily fish intake and children's risk of developing asthma was greater in children whose mothers had asthma compared to children of non-asthmatic mothers (p for interaction = 0.02). On the contrary, Calvani *et al.* (158) observed that the protective effect of increased oily fish intake during pregnancy against offspring food sensitisation was greater for children of non-allergic mothers compared to those of allergic mothers (p for trend = 0.002). However this was not the case for offspring inhalant sensitisations. It is not clear why these findings are different.

Moreover, in the prospective cohort study conducted by Romieu *et al.* (155), although a beneficial association was observed initially for the whole sample, after stratifying by breast-feeding, increased fish consumption during pregnancy decreased the risk of persistent wheeze at 6 years of age decreased among the non-breastfed infants whereas no protective effect was observed among the breastfed infants.

The large cohort studies conducted by Sausenthaler *et al.* (154, 156) 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. However the decrease in the study of Willers *et al.* (156) was greater than that in the study of Sausenthaler *et al.* (154) (43% vs. 25% respectively). This might be related to the fact that the study of Willers *et al.* (156) followed-up children for a longer period, allowing for manifestations of atopic disease to be revealed at the stage of clinical assessment. Willers *et al.* (156) also showed that there was a 72% decrease in doctor-diagnosed hay-fever in children born to mothers with higher oily fish intake (but not total fish intake) during pregnancy.

Across these five studies, the extent of the protective effect is highly variable. Fish intake resulted in decrease in childhood or infancy atopy which ranged between 25% and 95%. However, most decreases in atopic risk ranged between 40% and 80%. These five studies provide strong evidence of protective effect of maternal fish intake during pregnancy, however heterogeneous was the extent of the protective effect.

The effect of maternal fish intake during lactation on atopic outcomes in the infant

The last study of Table 1.5 conducted by Hoppu *et al.* (159) assessed maternal dietary intake one month after birth (during lactation) and atopic dermatitis development in the infant at four 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 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 explained by 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 (160, 161). The authors stated that maternal fish intake during pregnancy would have been more appropriate to investigate in relation to breast milk composition (159).

Summary

All five epidemiological studies investigating the effect of maternal fish intake during pregnancy on atopic or allergic outcomes in infants/children of those pregnancies concluded protective associations. However, the protective effect varied widely between 25% and 95% and this might be attributed to differences in study design i.e. confounding factors adjustments, statistical analysis, definition of atopic outcome in infants/children and their mothers, method of atopy evaluation, method of collecting dietary information, oily and/or total fish definition as well as categories of consumption frequencies used for comparisons. The one study investigating the effects of maternal fish intake during lactation did not observe any significant associations, suggesting that prenatal maternal fish oil supplementation (during pregnancy) might be more beneficial in association to reducing atopy risk in the offspring, as this is more likely to determine fatty acid composition of breast milk and thus provision of n-3 PUFA to the infant.

Table 1.5 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	Findings
Salam *et al* 2005 (157)	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

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Calvani *et al* 2006 (158)	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: -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): -Increased consumption of fish was associated with 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	Findings
Romieu *et al* 2007 (155) Antenatal Clinics in Manorca, Spain	462 pregnant women and their offspring (follow up at age 1 and 6 years) Prospective cohort	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	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 to 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 (154) 4 German cities	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)	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)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Willers *et al* 2007 (156) Aberdeen Maternity Hospital, Scotland	1212 pregnant women and their children followed up to age 5 years Prospective cohort	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: 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)
Hoppu *et al* 2005 (159) Finland	34 atopic mothers and their infants followed up to age 1 year	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 acid(s); SFA, saturated fatty acid(s); 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.

1.5.1.2 Studies investigating the effects of fish intake during infancy or childhood on atopic outcomes in those infants or children

Table 1.6 summarises all identified 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 (1950-2009) and Embase (1980-2009) databases performing and combining searches with the following keywords: fish intake, oily fish intake, pre-school children, 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 1.5.

Nine studies observed a beneficial effect of fish intake during childhood/ infancy and atopic outcomes in those children/infants (162-169). Two of the studies observed a negative effect of fish intake on childhood atopy (170, 171), and three studies observed no associations (172-175). All studies had large sample sizes, although there was a wide range of sample sizes amongst the studies.

The studies of Table 1.6 are discussed according to whether they observed a protective, negative or a null effect.

Studies showing a protective effect of fish

Study design

Of the studies that found a beneficial association between fish intake during infancy/ childhood and atopic outcomes, three were prospective cohort (164, 167, 175), two studies were case-control (162, 163), and four were cross-sectional (165, 166, 168, 169). The age range of children taking part in these studies was between 1 and 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 (162, 163, 165, 166) than others (164, 167, 168, 175). Two of the prospective cohort studies followed-up infants to age 4 years (164, 167), whereas Alm *et al.* (175) followed-up infants to age 1 year.

Exposure measures

Studies were inconsistent as far as exposure assessment is concerned. All three prospective cohort studies (164, 167, 175) determined the time point of fish introduction during the first year of life using a parental questionnaire. In addition Kull *et al.* (167) collected information on fish consumption frequencies. The cross-sectional study of Kim *et al.* (166) and Antova *et al.* (169) used a parental FFQ, that of Chatzi *et al.* (168) used a semi-quantitative FFQ and the other cross-sectional study used parental report of fish consumption without frequencies (165). The retrospective case-control study of Dunder *et al.* (163) used a 48-hour recall of intake and the case-control study of Hodge *et al.* (162) 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 repeated in different time points in the case of prospective cohort studies which have a long period of follow-up. In this way changes in the fish eating patterns of the children could be identified and related with allergy/atopy risk.

Oily fish consumption was recorded in only some of the studies (162, 168, 175). The rest of the studies recorded only total fish intake without specifying the types of fish. In the study of Hodge *et al.* (162) fresh oily fish consumption had a greater beneficial effect on current asthma than total fish consumption (74% versus 48% respectively). The 'Infants of Western Sweden' study of Alm *et al.* (175) collected information on the fish usually consumed and this was categorised into two different types of fish (lean and oily). The vast majority of the infants consumed lean fish, and in the univariate analysis it was shown that eating lean fish reduced eczema risk by 19% at one year of age (OR 0.81, 95% CI 0.68-0.97, $p = 0.025$), but the effect was lost in the multivariate analysis (175). However, Chatzi *et al.* (168) did not identify any significant association for any of the subgroups of fish included in their FFQ.

Confounding factors

The different studies controlled for different confounding factors. Kim *et al.* (166) and Antova *et al.* (169) adjusted for all other dietary information collected in the study. Alm *et al.* (175) performed a multivariate analysis, after identifying significant risk factors in the

univariate analysis, and adjusted for 'atopic reasons' for not having furry animals and for cow's milk allergy. Chatzi *et al.* (168) 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 in the studies of Hodge *et al.* (162), Nafstad *et al.* (164), Andreasyan *et al.* (165), Alm *et al.* (175), and Kull *et al.* (167), and passive smoking was controlled in the study of Antova *et al.* (169). Parental history of atopy and breastfeeding was controlled for in the studies of Nafstad *et al.* (164), Kull *et al.* and Chatzi *et al.* (168), and parents' allergy was controlled in the study of Antova *et al.* (169). Hodge *et al.* (162), Alm *et al.* (175), and Nafstad *et al.* (164) 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.

Outcome measures and findings

Atopic outcome definitions and assessment methods differed between studies. Three of the studies performed skin prick testing (SPT) (162, 165, 168) and one study determined specific IgE to identify sensitisation (167). In the study of Alm *et al*. (175) no clinical test or biochemical measurement of allergy was conducted. Clinical outcomes measured in all of the studies were assessed with parental questionnaires. However parents were not always asked for doctor-diagnosis. Clinical outcomes in all of the studies were generally well defined.

The reduction in atopy/allergy risk due to fish intake ranged between 22% and 80%. However, the risk reduction in most cases was between 50% and 60%, providing consistent evidence for the protective effects of fish consumption during childhood/ infancy on atopy/ allergy. The study of Antova *et al.* (169) showed that low fish intake, compared with higher intake, increased the risk of respiratory symptoms by 21% (current wheeze).

Nafstad *et al.* (164) observed a protective effect of introduction of fish early into diet on allergic rhinitis for the whole study population, but this protective effect remained significant only among children who were breastfed for more than 6 months, without parental hay-fever or asthma, with early life atopic eczema, or without an episode of lower respiratory tract infection during the first year of life. Similarly, in the other prospective cohort study of Kull *et al.* (167), although there was a dose-dependent reduction in risk of

atopic outcomes with increased fish consumption frequency, the protective effect was significant only for children without parental allergy. Additionally and in contrast to the study of Nafstad *et al.* (164), in the study of Kull *et al.* (167) 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 ≥ 9 months) and this was associated with a lower risk of eczema at 4 years of age (167). This agrees with the findings of Alm *et al.* (175) who found that the introduction of fish before 9 months of age had a protective effect on eczema at infants at one year, lowering the risk by 24% at 1 year of age. All the rest of the studies which identified a protective effect conducted their analysis for the study population as a whole.

Studies showing a negative effect of fish

Study design

Two studies were identified that found a negative effect of fish consumption on atopic/allergic outcomes in children/infants (170, 171). Both studies had a cross-sectional study design, measuring exposure and outcome at the same time point. This study design is not strong enough to infer causality. All sample sizes can be considered as large enough. Compared to the studies that identified a positive effect, these studies were conducted in older children.

Exposure measures

Both studies used a FFQ. The FFQ in the studies of Takemura *et al.* (171) was completed by parents. The study of Huang *et al.* (170), apart form the FFQ, also used a 24-h recall and compared the effect between quartiles of intake rather than consumption frequencies. Also, the study conducted by Huang *et al.* (170) included oily fish in the questionnaire.

Confounding factors

Takemura *et al.* (171) adjusted for parental atopy, and children's fruit and vegetable intake. Huang *et al.* (170) adjusted for the level of urbanisation and dietary factors in the multivariate analysis. None of these two studies adjusted for socio-economic factors. As mentioned previously, these are very important since fish intake may be associated with a specific lifestyle. In addition, a possible confounding factor could be parental beliefs about when specific food items should be introduced in their infants' diet, which can change

dietary initiation of a food item. For example, parents may avoid or promote the introduction certain foods if they believe that this could help prevent allergy development.

Outcome measures and findings

As far as the atopic outcome measures are concerned, in the study of Takemura *et al.* (171) questionnaires about atopy were answered by parents whereas in the study of Huang *et al.* (170) questionnaires were answered by children. Both studies were focused on clinical outcomes and included a component of doctor-diagnosis of atopic outcomes in their questionnaires. The study of Huang *et al.* (170) 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 premature form of the disease that might limit the revealing of clinical observation of atopy.

In the study of Takemura *et al.* (171), comparing children with current asthma and healthy children there were no differences in fish consumption frequencies. However, the increase in risk (12% for current asthma) was significant when comparing the effect of fish intake 1-2 times/month with that of fish intake 1-2 times/week. Also, the authors showed that the risk of current asthma increased with increasing fish intake (p for trend = 0.0349).

Although the univariate analysis Huang *et al.* (170) showed that higher oily fish intake was associated with higher prevalence of doctor diagnosed asthma, in the multivariate analysis this association was no longer significant. Also, no associations were found for total fish, seafood and shellfish intake and allergic rhinitis or asthma.

Studies showing no effect of fish

Study design

Of the three studies that did not identify any statistically significant asssociations between fish intake and atopic or allergic outcomes in infants/children, two of the studies were prospective cohort (173, 174) and one study was case-control (172). All studies were conducted in children older than 1 year of age. Hijazi *et al.* (172) collected fish intake data at age 12 years, Farchi *et al.* (173) at age 6-7 years and Wijga *et al.* (174) at age 2 years.

Exposure measures

All three studies used a parental/maternal FFQ. The study of Farchi *et al.* (173) 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.* (172) were not clearly defined in the questionnaire. What is more, the two prospective cohort studies (173, 174) 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.

Outcome measures and findings

As far as the outcome measures are concerned, the two prospective cohort studies (173, 174) assessed atopic outcome based on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaires completed by parents. The ISAAC questions were also used in the study of Hijazi *et al.* (172), but they were answered by the children themselves. Two of the studies (172, 174) reported results of univariate analysis. 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 be revealed. It has to be mentioned that the cross-sectional data recorded in 1980 during the case-control study, nested in the follow-up study of Dunder *et al.* (163), identified a null association between fish consumption and any atopic diseases (in 1980). However, as mentioned above, the retrospective follow-up showed a protective effect of fish. In the same study the cross-sectional data of 1986 were not presented.

Summary

Overall, the evidence from epidemiological studies investigating the effects of fish intake during infancy and childhood on atopic outcomes in those infants or children is inconsistent. However, the majority of these studies (nine of 14) showed a protective effect of fish intake during infancy or childhood on atopic outcomes in those infants/children. The reduction in atopy/allergy risk amongst these studies ranged between 22% and 80%. This could be attributed to the fact that studies differed in study design, control of confounding factors, exposure and outcome measure assessment. Also, it has to be underlined that some of the studies performed subgroup analysis (for example Kull *et al.* (167) and Nafstad *et al.* (164)) which may increase the risk of finding chance associations. There were only three studies that did not observe any associations (172-174), and two

studies (170, 171) that observed increased risk of atopy with higher fish consumption (12% increase of risk for current asthma (171)), although the study of Huang *et al.* (170) did not show any significant results in the multivariate analysis. Therefore, based on the evidence available from epidemiological studies, it cannot be clearly concluded with absolute certainty whether fish consumption during infancy or childhood can be protective towards atopic disease development in those infants/children, although a number of studies would support that conclusion.

Table 1.6 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	Findings
Hodge *et al* 1996 (162) Sydney, Australia	468 children aged 8-11 years Case-control	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	Unadjusted analysis: Total fresh fish (OR 0.50; 95% CI 0.27-0.92, $p< 0.05$) and oily fresh fish (OR 0.29; 95% CI 0.13-0.67, $p< 0.01$) associated with reduced risk of current asthma Adjusted analysis: Oily fresh fish associated with reduced risk of current asthma (OR 0.26; 95% CI 0.09-0.72, $p< 0.01$)
Hijazi *et al* 2000 (172) Saudi Arabia (rural and urban areas)	114 asthmatic and 202 non-asthmatic children; aged 12 years (from a population of 1444 Saudi-Arabian children) Case-Control	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

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Dunder *et al* 2001 (163) Finland (rural and urban areas)	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)	Dietary intake including fish intake (standardised to energy intake); in 1980 and 1986 48-h recall administered by nutritionists 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 (170) The 1st Nutrition and Health Survey in Taiwan	1166 adolescents aged 13-17 years Cross-sectional	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: age, levels of urbanisation FFQ variables used in the multivariate model: liver, deep-fried foods, oily fish, butcher's meat	• 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

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Takemura *et al* 2002 (171) The Tokorozawa Childhood Asthma and Pollinosis Study, Japan	Children aged 6-15 years: 1673 currently asthmatic and 22109 non-asthmatic Cross-sectional	FFQ including fish, completed by parents (Japanese Ministry of Health and Welfare) almost none 1-2 times/month 1-2 times/week ≥3-4 times/week	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). Increasing frequency of fish intake was associated with increased risk of current asthma (*p*-trend= 0.0349)
Antova *et al* 2003 (169) 25 areas in 6 Central and Eastern European Countries (Bulgaria, Czech Republic, Hungary, Poland, Romania, Slovakia)	20271 children aged 7-11 years Cross-sectional	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)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Farchi *et al* 2003 (173) Italian Studies on Respiratory Disorders in Children and the Environment (SIDRIA), part of the ISAAC	4104 children aged 6-7 years, followed-up for one year Prospective cohort	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	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

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Nafstad *et al* 2003 (164) Oslo Birth Cohort Study, Norway	2531 infants followed up to age 4 years Prospective cohort	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) 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) 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)
Wijga *et al* 2003 (174) The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort study, Netherlands	2978 children, aged 2 years; follow-up to 3 years old Prospective Cohort	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	Univariate analysis: Eating fish at least once a week was not associated with ever asthma, recent asthma or recent wheeze

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Andreasyan *et al* 2005 (165) Childhood Allergy and Respiratory Health Study Tasmania, Australia	499 children aged 8 years Cross-sectional	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 (166) All primary schools in Knivsta, Sweden	1014 children aged 5-14 years Cross-sectional	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)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Kull *et al* 2006 (167) Stockholm, Sweden	3619 infants followed up to age 4 years Prospective cohort	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 age 4 years • 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
Chatzi *et al* 2007 (168) Menorca, Spain	460 children aged 6.5 years Cross-sectional	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)	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

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Findings
Alm *et al.* 2009 (175) 'Infants of Western Sweden' Western Sweden	4921 infants aged 1year Prospective Cohort	Food frequency data including fish 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 (cod, haddock) Salmon Flatfish Mackerel or herring	Paternal report of eczema. Parental report of food allergy diagnosed by a physician at 6 and 12 months of age Multivariate analysis was performed for those risk factors that were significant in the univariate analysis: maternal eczema, sibling with eczema, bird in the home, introduction of fish before 9 months of age. Also adjusted for 'atopic reasons' for not having furry pets and for cow's milk allergy	• Multivariate analysis: -Introducing fish before 9 months of age reduced the risk of developing eczema at 1 year of age (OR 0.76; 95% CI 0.62-0.94, *p*= 0.009) -No influence of the type of fish (lean/oily) consumed • Univariate analysis: Usually eating lean fish reduced the risk of eczema at 1 year of age (OR 0.81; 95% CI 0.68-0.97, *p*= 0.025)- this was not significant in the multivariate analysis

OR, odds ratio; CI, confidence interval; PUFA, polyunsaturated fatty acid(s); 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; AHR, airway hyper-responsiveness; vs, versus.

1.5.2 Intervention studies investigating the effect of early fish oil exposure on atopic outcomes in infancy or childhood

In general, randomised controlled trials (RCT) or experimental/intervention studies are study designs that provide the highest level of testing of cause and effect. This section reviews the literature published to present on RCT of fish oil supplementation in pregnancy, lactation or infancy/childhood and allergic outcomes in infants/children of those pregnancies or those infants/children (Table 1.7 and Table 1.8). The studies were identified through Ovid Medline (1950-2009) and Pubmed (1950-2009) databases by searching with the following keywords: fish oil, fish supplements, fish-oil capsules, EPA, DHA, trial, maternal, pregnancy, lactation, atopy, allergy, asthma, eczema, dermatitis, hayfever, pre-school children, childhood, infancy.

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

Table 1.7 summarizes RCT of maternal fish oil supplementation during early life (pregnancy and lactation) and immune or allergic outcomes in the offspring during infancy or childhood; five such studies were identified. One study investigated the effects of n-3 PUFA supplementation during pregnancy and many scientific papers have been published on this study (22, 176-182), of which six (22, 176-179, 182) refer to clinical allergic outcomes in the offspring or immune markers that may modulate these outcomes (Table 1.7). 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 (183, 184) of which one (183) is included in Table 1.7. A 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 (185). The fourth trial, conducted by Olsen *et al.* (186) 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 up to present two relevant papers, one looking at the effects of the supplementation on the offspring during the first year of life (187) and the other at the effects of the supplementation during pregnancy on the immune system of the mother (188).

Study Design

The study by Dunstan and colleagues (22, 176-182) was conducted in Australia and was a double-blinded RCT starting at week 20 of pregnancy. The study of Krauss-Etschmann *et al.* (183) 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.* (185) 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.* (187) 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*. (186), 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.* (187) compared marine n-3 fatty acid supplementation with soy oil as placebo. The studies of Dunstan *et al.* (22, 176-182), Lauritzen *et al.* (185), and Olsen *et al.* (186) compared marine n-3 fatty acid supplementation with olive oil as placebo. However, in the study of Lauritzen *et al.* (185) 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.* (186) there was also a third group which was a control group and which received no oil capsules. Krauss-Etschmann *et al.* (183) 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.* (22) 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. The subjects' habitual dietary intake did not exceed two fish meals per week as assessed using a semi-quantitative FFQ prior to the study. In the study of Krauss-Etschmann *et al.* (183) subjects were healthy pregnant women (including both atopic and non-atopic subjects). Only women who did not use fish oil, folate and vitamin B12 supplementation after week 16 gestation were included in the study. Mothers that took part in the Danish study (185) were healthy and non-atopic. Estimation of their habitual LC n-3 PUFA (g/day) intake was conducted using a semi-quantitative 300 item FFQ and only women with an intake below the population median (< 0 .4 g/day) were randomized. 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.* (186) 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.* (187) both atopic and non-atopic women 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. Only women that planned to breastfeed their offspring were included in the study. At baseline (25 weeks of gestation) 3-day dietary diaries were conducted and PUFA nutrients were calculated. In both groups levels of EPA and DHA intake were 0.2g/day and 0.1 g/day respectively, while in the placebo group the LA intake was about 7 g/day (187).

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 between week 20 of gestation to delivery (22), between week 22 of gestation to delivery (183), and between 30 weeks of gestation to delivery (186). For one study supplementation occurred only postnatally, during the first 4 months of lactation (185) and for the study conducted by Furuhjelm *et al.* supplementation occurred perinatally, started in pregnancy (25^{th} week) and finished in lactation (average 3-4 months of breastfeeding) (187).

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.* (22) received 3.7 g/day of n-3 PUFAs with 56% as DHA and 27.7% as EPA. The control group received 4 g/day of olive oil. In the study of Krauss-Etschmann *et al.* (183) women received a lower dose of fish oils: 0.5 g/day DHA and 0.15 g/day EPA. Women in the study of Olsen *et al.* (186) received 2.7 g/day EPA plus DHA,

while the placebo group (one of the 2 control groups) received 4 g/day olive oil. In the trial of Furuhjelm *et al.* (187) the pregnant women assigned to the intervention group consumed 1.6 g/d EPA and 1.1 g/d DHA while the placebo group consumed 4.5 g/day soy oil containing mainly linoleic acid. Mothers in the study of Lauritzen *et al.* (185), were supplemented with 4.5 g/day of fish oil which provided 1.5 g/day of EPA plus DHA or with 4.5 g/day of olive oil. It has to be noted that the supplementation in the studies of Dunstan *et al.* (22), Furuhjelm *et al.* (187), and Olsen *et al.* (186) was in the form of capsules, in the study of Krauss-Etschmann *et al.* (183) in the form of powder stirred into a milk-based drink, whereas in the study of Lauritzen *et al.* (185) the fish oil was incorporated into muesli bars, home-made cookies and capsules.

Subject 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 EPA and DHA into the cell membranes of erythrocytes (178).

In the European multicenter study (183), there is no reference to subject compliance rates. However, this trial has published results on DHA and EPA incorporation in the mother and offspring (184). 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 she had missed. The drop-out rate was 13.18% (270 of the 311 recruited pregnant women completed the study) (184).

In the study conducted by Furuhjelm *et al.* (187) the overall dropout from gestation week 25 till delivery was 17%, however 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 at and 65 infants followed up in the control group. This means that the dropout in the fish oil group was 25% in the control 13%, while overall, the attrition rate was 19%. Authors did not comment on 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/placebo (187).

In the study of Olsen *et al.* (186) 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 three times, so that the researchers estimated amounts of capsules consumed (186).

Lauritzen *et al.* (185) 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 2.5 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%, $p = 0.020$) (185).

Confounding factors

In the study of Dunstan and colleagues, so as to minimise potential confounding factors at randomization, the groups were stratified by parity (no previous term birth child versus one or more), pre-pregnancy BMI, age and maternal allergy (allergic rhinitis or asthma). Results were adjusted for gender, parity and method of delivery (178). Dunstan *et al.* (22) 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.* (183), after randomization the women in different groups did not differ significantly in parity, height, weight at study entry, smoking habits, or social demographic characteristics. Also, data on dietary habits were obtained at study entry (184). However, data on their dietary intake were not 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 this 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 (reference Hungary) 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.* (186) did not control for any factors but randomisation was stratified by maternal habitual fish intake (low, medium and high).

Furuhjelm *et al.* (187) made adjustments in their analysis for allergic symptoms in children for the following factors: linoleic and arachidonic acid 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 (187).

Lauritzen *et al.* (185) did not control for confounder factors in their analysis. However they 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).

Findings of the studies

Findings of the Australian fish oil supplementation study (22, 176-182)

Maternal fish oil supplementation resulted in higher EPA and DHA status and lower AA status in cord blood erythrocytes (180) and in breast milk (179, 181). Cord blood plasma and urinary F2-isoprostane concentrations, considered to be markers of lipid peroxidation, were lower in the fish oil group (176), suggesting that maternal fish oil supplementation during pregnancy might be protective against oxidative stress in the infants of atopic mothers soon after birth. A number of immunologic effects of maternal fish oil supplementation during pregnancy were reported. These include significantly lower cord plasma IL-13 concentrations (178), a tendency towards lower cord blood mononuclear cell cytokine responses to allergens which was significant for IL-10 in response to cat allergen (22), and lower LTB_4 production and higher LTB_5 production by cord blood neutrophils (182). Cord plasma IL-13 and cord neutrophil LTB_4 production were inversely related to n-3 PUFA status. Although breast milk IgA, soluble CD14 and cytokines were not different between fish oil and control groups, IgA concentration was positively correlated with breast milk DHA (179). Denburg *et al.* (177) showed an altered cord blood hemopoietic progenitor phenotype in the fish oil group: there was an increased number of cord blood $CD34^+$ progenitors and an increased number of IL-5 responsive cord blood eosinophil/basophil colony forming units. The number of $CD34^+$ cells in cord blood significantly increased the risk of atopic eczema in infants at 1 year of age, and cord blood progenitor IL-5 responsiveness increased the risk of atopic eczema and recurrent wheeze significantly. Paradoxically, these findings suggest that fish oil supplementation during pregnancy may actually favour the development of atopic disease in the offspring. In

contrast to this conclusion, infants in the fish oil group were significantly less likely (OR 0.09) to have severe atopic dermatitis at 1 year of age and were three times less likely to have a positive SPT to egg at 1 year of age (OR 0.34) (22). It has to be noted that this study was not statistically powered to assess effects on clinical outcomes. Analysis at 2.5 and 5 years 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.

Findings of the European multicenter pregnancy supplementation study (183)

As mentioned above, 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 an increased proportion of DHA and EPA in maternal plasma at 30 weeks of gestation and at delivery and of DHA in cord blood plasma compared with placebo (184). What is more, fish oil supplementation increased the percentage of DHA in placenta phospholipids ($p = 0.008$). However, placental ARA was not significantly different between the groups (189).

Fish oil supplementation during pregnancy was associated with decreased mRNA expression of IL-4, IL-13 and CCR4 and decreased frequencies of NK cells and $CCR3^+$ $CD8^+$ T cells in cord blood (183). Moreover, mRNA levels of IL-1 and IFN-γ in maternal blood at delivery were decreased after fish oil supplementation. In contrast, mRNA levels of the regulatory cytokine TGF-β were higher in both maternal blood at delivery and in cord blood following maternal supplementation (183). Thus, it was shown that fish oil supplementation during pregnancy downregulates Th1 responses in the mother and Th2 responses in the foetus. These results are in line with the observations made by Dunstan *et al.* (178) of lower cord blood IL-13 concentrations after maternal fish oil supplementation. In addition, Krauss-Etschmann *et al.* (183) showed that the decrease in cord blood IL-13 mRNA levels was more pronounced in non-allergic mothers. No clinical assessments of the infants from this study have been reported.

Findings of the Danish follow up study (186)

Olsen *et al.* (186) conducted the supplementation trial in 1992 and related late pregnancy fish oil supplementation to prolonged gestation. After 16 years, they followed up the offspring of those mothers that 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 odds ratios for asthma (all types) and allergic asthma were both lower (by 63% and 87% respectively) in the fish oil group in comparison to the olive oil group (control). There was a lower prevalence of asthma (all types), atopic dermatitis or allergic rhinitis (by 57%) and of allergic asthma, atopic dermatitis or allergic rhinitis (by 69%) in the fish oil group compared to control. 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 results (186).

Findings of the Swedish pregnancy and lactation study (187, 188)

Warstedt *et al.* (188) showed that plasma phospholipid EPA and DHA increased in the fish-oil-treated women during pregnancy with similar changes seen in both atopic and non-atopic women. Lipopolysaccharide (LPS)-induced PGE_2 secretion from whole blood cultures was decreased in the majority of the fish oil group (188). 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 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 two groups ($p = 0.002$) and the decrease in PGE_2 secretion in the n-3 supplementation group was more pronounced among non-atopic women (but not significant). As far as secretion of LTB_4, chemokines and cytokines, no changes were observed with supplementation. 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 AA and negatively with changes in EPA, which was even stronger among the non-atopic (188).

Furuhjelm *et al.* (187) associated the fish oil supplementation during pregnancy and lactation (25 weeks gestation to 3-4 months lactation) with atopic clinical outcomes in the offspring at 6 months and 1 year of age. 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. 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 year of life were significantly lower in the fish oil group. In a regression analysis, after controlling for the 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 10 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 (187).

Findings of the 'Danish National Birth Cohort' (185)

Erythrocyte n-3 PUFA at 4 months were higher in infants whose mothers received fish oil during lactation compared to control. Differences in erythrocyte fatty acid composition between the groups were no longer evident at 2.5 years of age. The study was not powered to look at clinical outcomes, such as atopic sensitization, and no differences in atopic outcomes or in plasma IgE were observed between the groups. No 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 associations tended to be positive (185).

Summary

Dunstan *et al.* (178) showed that maternal fish oil supplementation resulted in higher n-3 PUFA status (higher EPA and DHA in cord blood erythrocytes) and lower n-6 PUFA status in the neonates. Krauss-Etschmann *et al.* (183) demonstrated that fish oil supplementation during pregnancy results in higher levels of DHA in both maternal and cord blood. These studies reported effects of maternal fish oil supplementation during pregnancy on cord blood immune markers (blood cytokine mRNA, plasma cytokines, LTB_4 production from neutrophils, cytokine production by mononuclear cells) and an altered cord blood hemopoietic progenitor phenotype. These immunologic effects might be expected to impact on allergic sensitization and on the development of atopic disease. Indeed, Dunstan *et al.* (22) reported 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 Danish study of Olsen *et al.* (186) identified that 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. The study conducted in Sweden (187, 188) involved fish oil supplementation during both pregnancy and lactation. Again, expected effects on n-3 PUFA status were observed and these were associated with differences in PGE_2 production. The latter might be expected to influence Th2 polarization. Indeed, 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. The Danish study of maternal fish oil supplementation during lactation (185) is the only one of these studies investigating immune outcomes in the offspring beyond birth. Infants of lactating mothers who received fish oil supplementation had a higher n-3 PUFA status at 4 months of age and IFN-γ production at 2.5 years of age was higher in the fish oil group, an observation which may reflect faster maturation of the immune system. This study did not assess clinical outcomes.

Thus, it is clear that fish oil supplementation during pregnancy and lactation results in higher provision of n-3 PUFA to the offspring and so in a higher n-3 PUFA status in the offspring. Early fish oil provision is associated with immunologic changes in cord blood and such changes may persist. These studies suggest clinical effects of early fish oil provision including reduced sensitization to common food allergens and reduced prevalence and severity of atopic dermatitis in the first year of life, again with a possible persistence until adolescence with a reduction in eczema, hay fever, and asthma. 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 to inform recommendations.

Table 1.7 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	Findings
Barden *et al* 2004 (176) Dunstan *et al* 2003 (178) Dunstan *et al* 2003 (22) Dunstan *et al* 2004 (179) Denburg *et al* 2005 (177) Prescott *et al* 2007 (182) Double-blinded RCT Perth, Australia	83 Atopic pregnant women n= 40 FO n= 43 Control	FO: 4 g fish oil/day (capsules) providing 3.7 g/day n-3 PUFA (56% DHA, 27,7% EPA) Control: 4 g olive oil/day (capsules) providing 66.6% oleic acid (<1% n-3 PUFA)	From week 20 of pregnancy until delivery	Cord blood: Plasma cytokine concentrations (IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, TNF-γ) Markers of APC function (HLA-DR expression and cytokine responses) Mononuclear cell cytokine responses to allergens and mitogen (IL-5, IL-10, IL-13, INF-γ) Plasma total IgE $CD34^+$ hemopoietic progenitors $CD34^+$ cell expression of cytokine (IL-5Rα, IL-3Rα) or chemokine receptors (CXCR4, CCR3) Eosinophil/Basophil colony forming units Leuktriene production by stimulated neutrophils In breast milk (3 days post-partum): immunomodulatory factors - sCD14, IgA, cytokines (IL-5, IL-6, IL-10, TNF-α and IFN-γ) Clinical outcomes at 1 year of age: SPT Symptoms of atopic disease (asthma, wheeze, food allergy, atopic dermatitis)	Maternal FO associated with: Lower cord blood plasma IL-13 ($p< 0.05$) HLA-DR expression on APC was not different between the groups Lower cord blood mononuclear cell cytokine responses (statistically significant only for IL-10 in response to cat allergen, p= 0.046) A higher percentages of cord blood $CD34^+$ cells ($p< 0.002$) Cord blood $CD34^+$ cell expression of cytokine and chemokine receptors was not different between groups More IL-5 responsive colony forming units ($p< 0.003$) Lower cord blood neutrophil LTB4 production ($p= 0.031$) No differences were observed in breast-milk IgA, sCD14, and cytokines between the groups Lower likelihood of a positive SPT to egg (OR 0.34, 95% CI 0.11-1.02, p= 0.055) Less severity in those infants with atopic dermatitis (OR 0.09, 95% CI 0.01-0.94, $p= 0.045$)

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Findings
Krauss-Etschmann *et al* 2008 (183) 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: 0.15 g EPA + 0.5 g DHA/day 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 until 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 Cord blood lymphocyte subsets	Maternal FO was associated with: Higher TGF-β mRNA in maternal blood at birth and in cord blood (both $p< 0.001$) Lower INF-γ and IL-1 mRNA in maternal blood at birth (both $p< 0.001$) Lower IL-4, IL-13, and CCR4 mRNA in cord blood (all $p< 0.001$) Lower proportions of NK and $CCR3^+$ $CD8^+$ T-cells in cord blood ($p< 0.001$ and $p< 0.04$, respectively)
Olsen *et al*. 2008 (186) 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: 4 g/day: 32% EPA, 23% DHA, provided 2.7 g/day marine n-3 PUFA 2. Control: olive oil capsules (4 g/day; 72% oleic acid, 12% LA) 3. No oil capsules	From week 30 of pregnancy until delivery	Offspring asthma-related diagnosis at 16 years of age: allergic asthma, asthma of mixed type, atopic dermatitis or allergic rhinitis Data taken from the National patient registry in Denmark	Maternal FO was associated with: -Lower risk of asthma (OR 0.37; 95% CI 0.15-0.92, $p= 0.03$) -Lower risk of allergic asthma (OR 0.13; 95% CI 0.03-0.60, $p= 0.01$) -Lower risk of asthma all types, atopic dermatitis or allergic rhinitis (OR 0.43; 95% CI 0.19-0.96, $p= 0.04$) -Lower risk of allergic asthma, atopic dermatitis or allergic rhinitis (OR 0.31; 95% CI 0.11-0.84, $p= 0.02$)

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Findings
Warstedt *et al.* 2009 (188) Furuhjelm *et al*. 2009 (187) 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 FO n=65 control	2 groups: 1. FO: 1.6 g EPA + 1.1 g DHA/day 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 breastfeeding)	Maternal outcomes: production of eicosanoids (PGE2 and LTB4), cytokines (IFNγ, IL-5, IL-6, TNF, IL8, IL10) and chemokines (CCL2, CCL3) by LPS-stimulated whole blood cultures Clinical examinations of infants: -Skin prick testing to cow's 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 Plasma specific IgE to egg/milk/wheat at age 3 and 12 months	From week 25 gestation until 1 week post-partum: -LPS-induced PGE2 secretion decreased in 64% of the FO supplemented mothers and increased in 77% of those in the control group (p= 0.002). -The decreased PGE2 production was more pronounced among non atopic (80%) than atopic mothers (69%) (not significant) -LPS-induced maternal cytokine and chemokine secretion was not affected. Maternal FO was associated with: -Lower prevalence of food allergy (2% vs. 15% in control group, p<0.05) -Lower prevalence of IgE-associated eczema (8% vs. 24% in control group, p<0.05). -Lower prevalence of any positive SPT (15% vs. 32% in control group, p= 0.04) -Lower prevalence of positive SPT to egg (12% vs. 29% in control group, p= 0.02) Logistic regression analysis revealed that maternal FO was associated with: -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 10 fold lower in the FO group compared to control group (OR=0.09, 95% CI 0.01-0.74, p<0.05)

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Findings
Lauritzen *et al* 2005 (185) Double-blinded parallel group RCT Denmark	Lactating mothers with fish intake below the population median: n= 37 FO n= 28 Control n= 26 Lactating women with fish intake in the highest quartile	3 groups: 1. 4.5 g FO/day providing 1.5 g/day of LC n-3 PUFA 2. Control: olive oil 3. Women with high fish consumption – no supplementation Supplements incorporated in muesli bars, home-made cookies, or capsules	First 4 months of lactation	Cytokine production in endotoxin-stimulated whole-blood cultures at 2.5 years of age Infant plasma IgE levels at 2.5 years of age Parental report of allergy diagnosis or tendency to allergy	Maternal FO was associated with: higher IFN-γ production (p= 0.034) and a higher ratio of IFN-γ to IL-10 (p= 0.019)

RCT, randomized control trial; OR, odds ratio; CI, confidence interval; SD, standard deviation; FA, fatty acid(s); PUFA, polyunsaturated fatty acid(s); 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; NK, natural killer cell(s); APC, antigen presenting cell(s); HLA-DR, human leukocyte antigen-DR; sCD14, soluble CD14; Eo/BCFU, eosinophil/basophil colony forming unit(s); Ig, immunoglobulin; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; TGF-β, transcription growth factor β; LPS, lipopolysaccharide; 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 1.8 summarizes the randomized controlled trials of fish oil supplementation during infancy/childhood on allergic outcomes in those infants/ children; five studies were identified. Six scientific papers have been published on the Childhood Asthma Prevention Study (CAPS), of which one describes extensively the study protocol (190). Table 1.8 summarizes the results of CAPS at 18 months (191, 192), at 3 years (193), and at 5 years of age (194, 195). Damsgaard *et al.* (196) also studied the impact of n-3 PUFA supplementation in infants. Studies done by Hodge *et al.* (197), Nagakura *et al.* (198), and Vaisman *et al.* (199) investigated the effect of fish oil supplementation in older children.

Study design

Both the CAPS (190) and the study of Hodge *et al.* (197) were conducted in Australia, the study of Nagakura *et al.* in Japan (198), the study of Vaisman *et al.* (199) in Israel, and the study of Damsgaard *et al.* (200) in Denmark. Infants (aged 6 months) who participated in the CAPS (190) were at high risk of developing asthma, whereas infants (aged 9 months) who took part in the Danish study (200) were not selected according to atopy. Children (aged 8 to 12 years) who participated in the study of Vaisman *et al.* (199) were healthy, but those in the studies of Hodge *et al.* (197) and Nagakura *et al.* (198) (aged 8 to 12 years and 4 to 17 years, respectively) were asthmatic.

All five trials were controlled, comparing fish oil with placebo. The placebos used in the studies varied. The CAPS (190) used capsules containing sunola oil, a monounsaturated fatty acid rich oil which also contained 7% n-6 PUFA, and dietary modification. Damsgaard *et al.* (200) used milk or formula without added oils. Hodge *et al.* (197) used capsules containing a mixture of palm, olive, and safflower oils and also replaced usual dietary fat by sunflower oil. Nagakura *et al.* (198) used capsules containing olive oil and Vaisman *et al.* (199) used canola oil blended in chocolate spread as a placebo. According to these strategies, the five studies provided different sources and amounts of n-6 PUFA to the control group. The mode of provision of fish oil and the dose of LC n-3 PUFAs given differed among the five studies (Table 1.8). In the CAPS tuna oil was given in capsules in an amount that was related to the age of the infant/child: the capsules were added into milk formula if infants had started bottle feeding before 6 months of age, or into formula and weaning foods after 6 months of age. At

the highest possible dose infants received 3.6 mg DHA and 0.8 mg EPA per kilogram of body weight (190). Also canola oil and canola oil-based margarine were used to lower the n-6 PUFA intake in the fish oil group, with the aim of achieving a dietary ratio of n-6 to n-3 fatty acids of 5. In the Danish study (200) liquid fish oil was used providing a mean daily intake of 571 mg EPA plus 381 mg DHA, although the range of intakes was wide, and it was advised to mix the oil into milk or formula. Hodge *et al.* (197) used fish oil capsules providing 1.2 g EPA plus DHA/day and replaced the usual dietary fat sources with canola oil and canola oil-based margarine. Nagakura *et al.* (198) used fish oil capsules with the number consumed adjusted according to body weight: thus daily intakes were in the range of 17 to 26.8 mg EPA and 7.3 to 11.5 mg DHA per kg body weight; the heaviest children consumed about 1.4 g EPA plus DHA/day. Finally, Vaisman *et al.* (199) provided fish oil in chocolate spread delivering 300 mg EPA plus DHA/day and in addition provided some canola oil. Three of the studies (197-199) were double-blinded, while the CAPS (190) was single-blinded, and the Danish study (200) was not blinded. The CAPS study included also a house dust mite (HDM) exposure modification as a separate arm (190).

The duration of supplementation varied between the studies: 3 months (199, 200), 6 months (197), 10 months (198), 5 years (190). Finally, 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 bottle feeding (or 6 months of age) to 5 years of age (190). The study of Damsgaard *et al.* (200) supplemented infants between months 9 and 12 of life. Hodge *et al.* (197), Nagakura *et al.* (198), and Vaisman *et al.* (199) conducted the intervention on children of a wide age range (8-12, 4-17, and 8-12 years of age, respectively). The differences in duration and dose of fish oil supplementation and in the ages of study subjects among the studies makes direct comparison difficult and may be a source of heterogeneity of findings.

Sample size, subject follow-up and compliance

Regarding the sample size, in the CAPS study it is possible that failure to show a clinical benefit resulted from lack of power (194). In the study of Damsgaard *et al.* (200), it was recognized that sample size was small and that the rate of completion with blood samples was low. Finally, in the study conducted by Hodge *et al.* (197), authors suggested that significant changes in TNF-α production may have had been detected if the sample size was larger.

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.* (200), 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.* (197) 6 children out of 45 dropped out at baseline (13% withdrawal). Vaisman *et al.* (199) did not have any dropouts. In the study of Nagakura *et al.* (198) only one child out of 30 dropped out.

In the CAPS, the moderate compliance rates might be related to the no association effect of the intervention on allergic outcomes at 3 and 5 years of age. In the CAPS, compliance was assessed my 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 (194). 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%, $p = 0.004$).

Damsgaard *et al.* (200) asked volunteers to return remaining bottles and report any waste. Damsgaard *et al.* (200) 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 teaspoons/day of the oil supplement into milk or formula). Hodge *et al.* (197) 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.* (199) 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.* (198) 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) (190). The clinical setting (hospital) in the study carried out by Nagakura *et al.* (198), and the fact that subjects stayed in the hospital ward for almost 85% of the intervention, was ideal as the environment was controlled both for nutrients and allergens. 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 18:2n-6 to 18:3n-3 of about 8:1 (200).

Hodge *et al.* (197) 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, as with the studies of Nagakura *et al.* (198) and Vaisman *et al.* (199), the study of Hodge *et al.* (197) did not control for fish intake or for any other confounding factors in their analyses.

Outcomes measures

The CAPS assessed clinical outcomes of allergic diseases in children at three different time points (18 months, 3 years, and 5 years of age) (194). 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 1.8). Clinical outcomes were also measured in the study conducted by Hodge *et al.* (197) in which lung function, atopy and asthma severity were assessed. Asthma severity and lung function were assessed in the study of Nagakura *et al.* (198). In contrast, blood immune markers were determined, instead of clinical outcomes, in the trials conducted by Vaisman *et al.* (199) and Damsgaard *et al.* (200). Blood immune markers were also determined in the CAPS study and in the study of Hodge *et al.* (197).

Findings of the studies

Findings of the CAPS (190-195)

In the CAPS, Alqmvist *et al.* (195) reported that dietary and plasma LC n-3 PUFA 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. At 18 months of age there was decreased prevalence of wheeze in the fish oil group and higher plasma n-3 PUFA levels were associated with lower bronchodilator use, irrespective of supplementation group (191, 192). Follow-up at 3 years of age suggested fish oil supplementation from infancy to childhood could reduce allergic sensitization and airway disease at early age, as fish oil supplementation reduced cough, but not wheeze (193). 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 was no significant effect of fish oil intervention on any of the clinical outcomes relating to lung function (194), allergy (194), or asthma (195). The CAPS (194) 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. It should be noted that the infants who 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 PUFA on the incidence of any allergic outcome at 5 years of age may be related to suboptimal adherence to and/or implementation of intervention (50% and 56% compliance in the intervention and control group, respectively), as well as to the dose of fish oil supplementation, loss to follow-up and lack of power.

Findings of Damsgaard et al. (200)

Damsgaard *et al.* (200) showed that fish oil increased erythrocyte n-3 PUFA status. There was a borderline significant effect of fish oil on IFN-γ production by whole blood cultures, which increased, but there were no other significant effects on markers of innate immunity or inflammation, although there was a non-significant tendency of a reduced IL-10 production. These effects could indicate a decrease in Th2 responses, with possible implications for the development of allergies (185, 196), but clinical outcomes were not assessed.

Findings of Hodge et al. (197)

In the study of Hodge *et al.* (197) fish oil supplementation increased plasma phospholipid n-3 PUFA status. TNF-α production by isolated mononuclear cells decreased significantly in the fish oil group but was not different from that in the control group at the end of the intervention. There was no effect of fish oil on lung function or asthma severity.

Findings of Nagakura et al. (198)

Nagakura *et al.* (198) showed that fish oil increased plasma EPA status and significantly reduced asthma severity score (by about 70% by month 10) and improved lung function in children (the provocative concentration of acetylcholine causing a 20% fall in the forced expiratory volume in 1 second was increased from an average of 980 µg/mL at baseline to an average of 1850 µg/mL at month 10).

Findings of Vaisman et al. (199)

Vaisman *et al.* (199) found that long term supplementation of moderate amounts of n-3 fatty acids to the diets of children increased the production of all cytokines measured (TNF-α, IL-1β, IL-6, IL-10, IL-1rα) in both unstimulated and LPS-stimulated mononuclear cell cultures. Clinical outcomes were not assessed. No baseline data on population characteristics or 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.

Summary

Provision of long-chain n-3 PUFA in the form of fish oil to infants or children increases the status of those fatty acids in plasma (191, 193-195, 197) and blood cells (196). Fish oil consumption may induce effects on the immune system in infants (196) and older children (197, 199). In children with asthma, fish oil did not affect lung function or asthma severity in the study conducted in Australia (197), but significantly improved lung function and asthma severity in the study conducted in Japan (198). These latter two studies had similar sample sizes. The study of Nagakura *et al.* (198) used a lower dose of n-3 PUFA than that of Hodge et al. (197), and so this does not explain the differences in outcome observed between these two studies. The study of Nagakura *et al.* (198) was of longer duration than that of Hodge *et al.*

(197) and did not identify a significant effect of fish oil on asthma score or lung function until month 6 of the intervention; the study of Hodge et al. (197) was of 6 months duration. The studies of Damsgaard *et al.* (196) in healthy infants and of Vaisman *et al.* (199) in healthy children did not report clinical outcomes. The CAPS, conducted in infants at risk of allergic disease, is the largest of these studies and involved the longest period of supplementation. This study reported some protective effects of fish oil in these infants at 18 months and 3 years of age, but these effects did not persist until 5 years at age (194, 195). Lack of persistence may be due to reduced compliance over time, loss to follow-up, lack of power, or the presence of confounding factors.

Thus, it is clear that fish oil supplementation during infancy or childhood results in higher n-3 PUFA status in those infants or children. Such early fish oil provision may be associated with immunologic changes in the blood but it is not clear if these are of clinical significance and whether they persist. Fish oil supplementation in infancy may decrease the risk of developing some manifestations of allergic disease, but this benefit may not persist as other factors come into play. It is not clear whether fish oil can be used to treat children with asthma as the two studies conducted to date (197, 198) give divergent results. Further studies are needed to identify immunologic and clinical effects of fish oil in infants and in children and to identify protective and therapeutic effects and their persistence.

1.5.3 Final summary and conclusions of the systematic review

It is considered that n-3 PUFA will protect against atopic sensitization and against the clinical manifestations of atopy. Evidence to examine this has been acquired from epidemiologic studies investigating associations between fish intake in pregnancy, lactation, infancy, and childhood and atopic outcomes in infants and children and from intervention studies with fish oil supplements in pregnancy, lactation, infancy, and childhood and atopic outcomes in infants and children All five epidemiological studies investigating the effect of maternal fish intake during pregnancy on atopic or allergic outcomes in infants/children of those pregnancies concluded protective associations. One study investigating the effects of maternal fish intake during lactation did not observe any significant associations. The evidence from epidemiological studies investigating the effects of fish intake during infancy and childhood on atopic outcomes in those infants or children is inconsistent, although the majority of the studies (nine of 14) showed a protective effect of fish intake during infancy or childhood on atopic outcomes in those infants/children. Fish oil supplementation during pregnancy and lactation or during infancy of childhood results in a higher n-3 PUFA status in the infants or children. Fish oil provision to pregnant women is associated with immunologic changes in cord blood and such changes may persist. Studies performed to date indicate that provision of fish oil during pregnancy may reduce sensitization to common food allergens and reduce prevalence and severity of atopic dermatitis in the first year of life, with a possible persistence until adolescence with a reduction in eczema, hay fever, and asthma. Fish oil provision to infants or children may be associated with immunologic changes in the blood but it is not clear whether these are of clinical significance and whether they persist. Fish oil supplementation in infancy may decrease the risk of developing some manifestations of allergic disease, but this benefit may not persist as other factors come into play. It is not clear whether fish oil can be used to treat children with asthma as the two studies conducted to date give divergent results. Further studies of increased long-chain n-3 PUFA provision during pregnancy, lactation, and infancy are needed to more clearly identify the immunologic and clinical effects in infants and children and to identify protective and therapeutic effects and their persistence (103).

Table 1.8 Fish oil supplementation during infancy/ childhood and allergic outcomes in those infants/ children

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Findings
Mirhshahi *et al* 2003 (191) Mihrshahi *et al* 2004 (192) CAPS 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 376 Children had blood taken	4 groups: 1. HDM reduction, placebo supplement 2. No HDM reduction, placebo supplement 3. HDM reduction, FO supplement 4. No HDM reduction, FO supplement FO group: 500 mg/day tuna oil capsules (37% n-3 PUFA, 6% n-6, 24% MUFA, 28% SFA, 5% minor FA), plus canola margarines and oil (16% n-6, 40% n-9, 6% n-3 PUFA)	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 PUFA, 82% MUFA, 9% SFA, 1.7% minor FA), plus margarines and oil (40% n-6, 20% n-9, 1.2% n-3) Outcomes at 18 months of age	At age 18 months: Total serum IgE Lymphocyte cytokine responses to allergen stimulation SPT Parental questionnaire: wheeze, cough, asthma history, eczema Clinical assessment for eczema	High n-3 PUFA exposure tended to lower serum IgE FO was associated with 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 High n-3 PUFA exposure was associated with a reduction in 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) After adjusting for breastfeeding and smoking during pregnancy, the highest n-3 PUFA exposure was only protective for bronchodilator use (OR 0.46, 95% CI 0.30-0.71, p< 0.0001)
Peat *et al* 2004 (193) CAPS As above	526 Children of 616 completed the study at 3 years of age	As above	As above Outcomes at 3 years of age	At 3 years: Total serum IgE SPT Parental questionnaire: wheeze, cough, asthma history, eczema Clinical assessment for eczema	FO was associated with a 10% reduction in the prevalence of cough (95% CI, 3.7-16.4, p= 0.003) in atopic children

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Findings
Marks *et al* 2006 (194) Almqvist *et al* 2007 (200) CAPS As above	516 Children of 616 completed the study at 5 years of age	As above	As above Results at 5 years of age	At 5 years: Total serum IgE SPT Parental questionnaire: wheeze, cough, asthma history, rhinitis, eczema Clinical assessment for eczema Spirometric lung function, respiratory system resistance measurements	FO supplementation did not have any effect on the outcome measures at 5 years of age
Damsgaard *et al* 2007 (200) Randomized, unmasked 2 x 2 factorial design Copenhagen, Denmark	64 Healthy Danish infants Cow's milk n= 13 FO n= 20 Control Infant Formula n= 20 FO n= 11 Control	4 Intervention groups: Cow's milk and FO Cow's milk only Infant formula and FO Infant formula only Mean fish oil consumption 3.4 mL/day (571 mg EPA and 381 mg DHA)	Between 9 and 12 months of age	Plasma IgE, CRP, sIL-2R concentrations Production of TNF-α, IFN-γ, and IL-10 in whole-blood cultures Fecal IgA at 10 months of age	FO supplementation: Increased induced IFN-γ production (p= 0.05) and tended to decrease IL-10 production (p= 0.08)

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Findings
Hodge *et al* 1998 (197) 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 PUFA 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	Circulating eosinophil numbers Production of TNF-α by stimulated blood mononuclear cells Lung function (AHR; FVC, FEV1) SPT Asthma severity (record of expiratory flow rate, symptoms, medication)	FO decreased TNF-α production compared to baseline (p= 0.026) but the magnitude of change between FO and control groups was not significant (p= 0.075)
Nagakura *et al* 2000 (198) 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) Lung function (acetylocholine challenge; FEV1)	FO was associated with: Decreased severity of asthma at 6, 7, 8, 9, 10 months after administration compared to baseline (p< 0.05) Improved lung function (lower responsiveness to acetylcholine) at 6, 8, 10 months after administration (p< 0.03)
Vaisman *et al* 2005 (199) 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	Cytokine production (IL-1β, TNF-α, IL-6, IL-10, IL-1rα) by unstimulated and LPS-stimulated blood mononuclear cells	FO supplementation associated with higher cytokine production in unstimulated and stimulated cultures

RCT, randomized control trial; CAPS; Childhood Asthma Prevention Study; OR, odds ratio; CI, confidence interval; FA, fatty acid(s); PUFA, polyunsaturated fatty acid(s); MUFA, monounsaturated fatty acid(s); SFA; saturated fatty acid(s); 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; AHR, airway hyper-responsiveness; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; LPS, lipopolysaccharide, vs., versus.

2 Salmon in pregnancy study (SIPS): aims, research hypotheses, study design and baseline characteristics of participants

2.1 Aims of the study and research hypotheses

There is a theoretical basis by which increased early exposure to long chain (LC) n-3 polyunsaturated fatty acids (PUFA), as found in oily fish and fish oils, should lower the risk of atopy and its manifestations including asthma. The literature review in Section 1.5 indicates that the effects of maternal fish oil supplementation during pregnancy on the immune system of the mother and the offspring, as well as on atopic disease development, have not been clearly demonstrated, although there is some evidence for benefits. However, epidemiological studies provide a fairly strong evidence base that fish consumption by pregnant women is protective against atopy development in their offspring (103). While it is widely considered that this is because fish provide LC n-3 PUFA, fish also provide other nutrients such as vitamin D and selenium. It is possible that it is these nutrients or the combination of these nutrients with LC n-3 PUFA that is important. To examine this further early intervention with fish is required.

It is recommended that pregnant women and women of reproductive age should consume oily fish but not more than two portions weekly, due to of the presence of contaminants in some fish (11). Other population subgroups are recommended to consume up to four portions of oily fish per week, or more if the individual wishes to do so (2). The level of contaminants in oily fish can be greatly reduced by aquaculture using feed resources that are low in contaminants.

There are no published intervention trials with fish during pregnancy. The Salmon in Pregnancy Study (SIPS) is the first single blind (investigator) randomised controlled trial with oily fish (in this case farmed salmon) in pregnant women. 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. SIPS is registered on the clinicaltrials.gov website with identifier number NCT00801502, and official title: 'The Effects of Oily Fish in Pregnancy on Markers and Manifestations of Allergic Diseases in Infants at Risk of Atopy'.
(http://clinicaltrials.gov/ct2/show/NCT00801502?term=sips&rank=2).

The overarching SIPS hypothesis, as stated on clinicaltrials.gov, is 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 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 measure of SIPS, as stated on clinicaltrials.gov is:

- Omega-3 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 measure of SIPS, as stated on clinicaltrials.gov are:

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

The core SIPS investigators were:

Professor Philip P. 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 Maria Vlachava, MSc	PhD student and Nutritionist
Miss Lefkothea-Stella Kremmyda, MSc	PhD student and Nutritionist

The present thesis presents data from only part of SIPS. The aims of the work described in this thesis are:

a) 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 is at a risk of developing atopic disease;
b) to investigate the effect of increased oily fish consumption on maternal blood and tissue LC n-3 PUFA status;
c) to investigate the effect of increased oily fish consumption on maternal immunity, which may potentially influence the developing foetal immune system and atopy risk of the offspring.

The research hypotheses examined on this thesis are:

that increased consumption of salmon during pregnancy by women with high risk of having an atopic offspring will:

a) increase maternal LC n-3 PUFA intake;
b) increase maternal LC n-3 PUFA status;
c) alter maternal immunity, which may potentially influence the developing foetal immune system, in a way that would decrease atopy risk for the offspring

The work addressing each of these three research hypothesis is described, and the findings presented and discussed, separately in Chapters 3, 4 and 5, respectively.

The work described presented in this thesis was conducted by me, L.-S. Kremmyda, as a member of the SIPS research team. The tasks described here were primarily my responsibility, although their completion required collaboration with the activities of the rest of the SIPS investigators and I acknowledge them for this. In addition, I was involved to an extent in other activities until the end of the study and these activities and their results are not described in this thesis. Briefly, other activities that I performed but were not my primary responsibility (and so are not reported here) were: making maternal anthropometry measurements including body composition using bioelectrical impedance; preparation of maternal erythrocytes, plasma and serum to be analysed by other research centres (for contaminant, antioxidant, vitamin, hormone, amino acid, oxidative stress marker, and inflammatory marker analyses); preparation of placenta and umbilical cord

tissue and umbilical cord vein and artery for immunohistochemistry; processing of umbilical cord blood for anlyses including fatty acids and immune markers; collection of urine, faecal (maternal and infant) and breast milk samples at various time points during clinics and home visits; administration of infant food frequency questionnaire at 6 months of age.

2.2 Study design

A hundred and twenty three (123) pregnant women whose baby had a high risk of being atopic 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. The study was single blind (investigator only), randomised and controlled (vs. habitual diet).

Eligibility criteria for inclusion in the study were:

1. In the early stages of pregnancy
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 foetus
4. Not consuming fish oil supplements
5. Aged between 18-40 years.

Exclusion criteria were:

1. Habitual consumption of oily fish (> 2 portions 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 (NSAID) (e.g. aspirin, diclofenac, ibuprofen, voltarol)
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.

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

2.3 Subject follow-up and data collection

Subjects taking part in the study were seen at 13, 19-20, 32-34, and 38 weeks of pregnancy and at several time points after they had given birth. At 13 weeks gestation subjects 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, pregnant women had received an initial information sheet about the SIPS (Appendix A), along with their appointment notes sent by the SWS UU. Subjects were initially approached by a researcher of the SIPS team and were asked if they would like to hear more about the study and be screened for eligibility. Subjects who agreed to be screened were asked to sign a consent form for initial screening (Appendix B). Initial screening included a health and lifestyle questionnaire administered by the researcher including questions on eligibility criteria, measurement of weight and height (Appendix C), and body composition measurement performed by bioelectrical impedance.

Subjects who were eligible for the study were provided with a second information sheet (Appendix D), the study was further explained to them and their intention to take part in the study irrespective of randomization group was confirmed. Subjects who were interested in the study but had a preference for either of the two groups were not included in the study. At 15 weeks of pregnancy subjects were phoned to be reminded about the study, discuss any concerns and confirm their next appointment at 19-20 week pregnancy at SWS UU, and they were randomly assigned in one of the two groups, intervention or control. A randomisation plan was created through the website http://www.randomization.com, which randomises 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.

The overall SIPS study was powered according to two outcomes: fatty acid composition of maternal plasma phospholipids and infant sensitisation to at least one common allergen by skin prick testing (SPT). The power calculations were based initially on 50 subjects per group, however because of anticipated withdrawals, recruitment target was raised to 60 subjects per group so as to have approximately the number of women (50) in each group that would achieve statistical significance at the end of the intervention. Specifically, a sample size of 50 per group was anticipated to have a 99% power to detect differences in maternal plasma eicosapentaenoic acid (EPA) phospholipids with $p < 0.05$ (90% power to detect with $p < 0.001$). A sample size of 50 per group would have a 70% power to detect differences in infant sensitisation to allergens (by SPT) with $p < 0.05$ (Appendix E)

Subjects attended the 19-20 week appointment in the fasted state. They were asked to sign the consent form for participating in the SIPS (Appendix F). This appointment included an ultrasound scan, weight measurement and body composition analysis with bioelectrical impedance, a researcher-administered health and lifestyle questionnaire (Appendix G), blood collection (see section 4), urine collection, and administration of a food frequency questionnaire (FFQ) covering the past three months (Appendix H). Women were given breakfast after blood sample collection and before the FFQ was administered. Subjects in the intervention group were 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 I). Subjects in the control group were provided with a general cook book including information on healthy eating during pregnancy, and a fish diary (Appendix J) to record their fish consumption (type, way of cooking, portion size).

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 measurements.

The health and lifestyle questionnaire was administered by an appropriately trained member of the SIPS team at 13, 20, 32-34, and 38 weeks of pregnancy. This included questions on medication, use of nutritional supplements/vitamins/minerals in the previous 4 weeks, physical activity, 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 C, G, K, L). Physical activity questions incorporated in the health and lifestyle questionnaire 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 past three months from the time point of the interview. The questions were adopted form the SWS questionnaire for estimating physical activity, which has been used before on women in Southampton (201-203).

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, 32-34, and 38 weeks gestation. This is a bioelectrical impedance device using multi-frequency 8-electrodes to measure body composition. This method enables measurement of impedance per segment – in other words for the whole body, right leg, left leg, right arm or left arm. The results of body composition measurement are not included in this thesis.

Midwives at the Princess Anne Hospital, Southampton and the rest of the 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 store the whole placenta and umbilical cord at 4˚C after delivery (see section 4). They were asked to contact the SIPS team once a sample was collected.

Subjects were followed up at 3 months post-partum when the FFQ was administered to them by an appropriately trained nutritionist during home visits.

Finally, both the mother and the father of the infant who attended the clinic at 6 months post-partum were skin prick tested to specific allergens. Skin prick test (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 a negative control (glycerine diluent) (Soluprick SQ, ALK Abello). A drop of each allergen and control was introduced at 90° into the epidermis using a lancet, without drawing blood. A wheal diameter of ≥3 mm greater than the negative control was

considered positive 15 minutes after the application of the allergen extracts. Table 2.1 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.

Table 2.1 Allergens used in skin prick testing (SPT)

Type	Strength
Negative Control	Glycerine diluent
Positive Control	10 mg/ml
Dermatophagoides pteronyssinus	10 Hep
Felis domesticus (cat)	10 Hep
Canis familiaris (dog)	10 Hep
6 Grass mix	10 Hep
3 Tree mix	10 Hep
Aspergillus fumigatus	1:20 w/v
Alternaria alternata	1:20 w/v
Cladosporium herbarum	1:20 w/v
Penicillum notatum	1:20 w/v

2.4 Study salmon

The salmon used in the study were farmed in Norway using high quality feed ingredients in order to be low in contaminants (dioxins and dioxin-like polychlorinated biphenyls (PCB), dichloro diphenyl trichloroethanes (DDT) and heavy metals (arsenic, cadmium, mercury, lead)). 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 it was shipped to Southampton and stored at -30˚C (Figure 2.1). The salmon were delivered to the intervention group, on a monthly basis, with enough to cover intake of two portions (150 g/portion) per week by the pregnant women and their partners. After delivery salmon were stored in home freezers.

Figure 2.1 Vacuum sealed salmon as delivered to the pregnant women

Frozen portions of salmon were analysed by the National Institute for Nutrition and Seafood Research, Norway. Thirty (30) portions of frozen portions were randomly sampled for analysis. The individual portions were pooled into 3 samples of 10 portions each for analysis of nutrient and undesirable content. Table 2.2 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 2.3 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 2.4 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 far less than the provisional tolerable weekly intake (see Table 2.6).

Table 2.2. 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 SFA	**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 MUFA	**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 PUFA	**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 PUFA	**22.0 ± 0.2**	**23.7 ±1.5**	**3.56**
Sum of total PUFA	**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 2.3. Vitamin, mineral, total protein and total fat content of tailored salmon (mean ± standard deviation; n = 3)

Vitamins	***µg/g 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 2.4. 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-PCB	0.35 ± 0.06 pg WHO TEQ/g	52.5 pg
Sum of DDT	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

PCB, polychlorinated biphenyl(s); dl, dioxin like; DDT, dichloro diphenyl trichloroethane(s); WHO TEQ, World Health Organisation toxic equivalent

Table 2.5 shows the contribution of two portions of study salmon to the recommended intake of EPA plus DHA (11), vitamin D (Reference Nutrient Intake; RNI) and selenium (RNI) (18) in the salmon group on a weekly basis. It can be seen two portions of the study salmon per week would provide more than the recommended weekly intake of EPA plus DHA (3460 mg/week or 494 mg/day), and would also contribute significantly towards achieving the recommendation for vitamin D (in pregnant and lactating women) and selenium. The mean daily intake of vitamin D in women aged 19-64 is below the RNI. Mean selenium intake among women 19-64 years old in the UK has been found to cover 72% of the RNI (which is 60 µg/day), and it was shown that 52% of women aged 19-64 had selenium intake below the Lower Reference Nutrient Intake (LRNI) which is 40 µg/day (13). Unfortunately, the most recent report of the National Diet and Nutrition Survey (2008/2009) (13) does not include data on EPA and DHA intake and estimated intake figures were taken from previous reports (12, 14, 15).

Table 2.5 Calculated contribution of two portions of study salmon to LC n-3 PUFA, vitamin D, and selenium recommended 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 (11)	> 450 mg	> 3150 mg	1730 mg	3460 mg	109%	< 200 mg but probably much less than this (12, 14, 15)
Vitamin D[1]	10 µg	70 µg	14 µg	28 µg	40%	2.7 µg[3]
Selenium[3]	60 µg	420 µg	43 µg	86 µg	20%	39 µg[3]

[1] Vitamin D Reference Nutrient Intake (RNI) for pregnant and lactating women (18)
[2] Selenium Reference Nutrient Intake (RNI) for 19-50 year old women (18)
[3] According to NDNS 2008/2009 (13)

Table 2.6 presents the calculated total intake of selected contaminant compounds from consumption of two 150 g portions of the tailored salmon per week. The calculated weekly contaminant intake expressed as per kg body weight is much lower than the provisional tolerable weekly intake (PTWI) (expressed per kg of body weight per week) established by the Joint Food and Agricultural Organisation/World Health Organisation Expert Committee on Food Additives (204, 205).

Table 2.6. 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	Intake from study salmon per week[1]	Intake from study salmon per Kg body weight per week[2]	PTWI (per Kg body weight per week)
Sum of dioxin and dl-PCB	105 pg	1.3-1.75 pg WHO TEQ	14 pg WHO TEQ
Sum of DDT	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; PCB, polychlorinated biphenyl(s); dl, dioxin like; DDT, dichloro diphenyl trichloroethane(s); 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

2.5 Progress of the study and subject follow-up

Figure 2.2 presents the flow of pregnant women into the two groups. In total 692 pregnant women were sent the initial information sheet about SIPS along with the appointment letter for their 13 week gestation ultrasound scan; these women were randomly selected over the 12 month recruiting period. Out of those women, 377 were screened for eligibility for SIPS, after signing the consent form for initial screening. Subsequently, a total of 315 women were not screened for eligibility. The reasons for which they were not screened can be seen on Figure 2.2, with the major reason (n = 241) being the lack of interest in the study.

Out of the women who expressed an interest and were screened (n = 377), 166 were eligible and agreed to be randomly assigned to either the control or intervention group, and 211 women were ineligible. The reasons for which women were not eligible can also be seen on the figure, with the main one being the absence of atopy for the mother, father or sibling. Out of those subjects who were eligible (44% of the screened), only 123 women (74% of the eligible) were randomised at 15 weeks gestation and enrolled onto SIPS at 20 weeks gestation. The rest of the women, although eligible, were either women who were randomised at 15 weeks gestation but not enrolled onto SIPS at 20 weeks (reasons given on figure), or were not randomised at all because of mainly not being interested in the study any more when contacted at 15 weeks gestation.

Figure 2.3 presents the progress of the study throughout its different stages. A total of 123 women were recruited onto SIPS after signing the SIPS consent form. All 123 subjects had their 20 week fasting appointment, during which they were randomly assigned to the control (n = 61) or the salmon arm of the study (n = 62). This number of subjects was slightly greater than the target of recruitment (n = 120). Before delivery, 9 subjects withdrew from the salmon group and 7 from the control group. Thus, up to delivery, the withdrawal rate was 14.5% for the salmon arm and 11.5% for the control arm, and 13% for the total of the subjects. The reasons for subject withdrawal from the study and at which time point they withdrew can be seen on Figure 2.3. A total of 111 subjects attended the 34 week appointment, and 91 subjects attended the 38 week appointment, while 14 subjects delivered before the 38 week appointment. Finally, birth samples were collected from 101 subjects. Between delivery and the 3 month post-partum visit 1 subject withdrew from the salmon group and 5 from the control group. A total of 86 3 month visits were completed

and 2 subjects were interviewed on the phone. Infants were followed up at 6 months of age for the development of eczema, asthma and allergen sensitisation. A total of 86 6 month infant appointments were completed (n = 47 salmon, n = 39 control). A total of 86 mothers and 76 fathers were skin prick tested at that appointment.

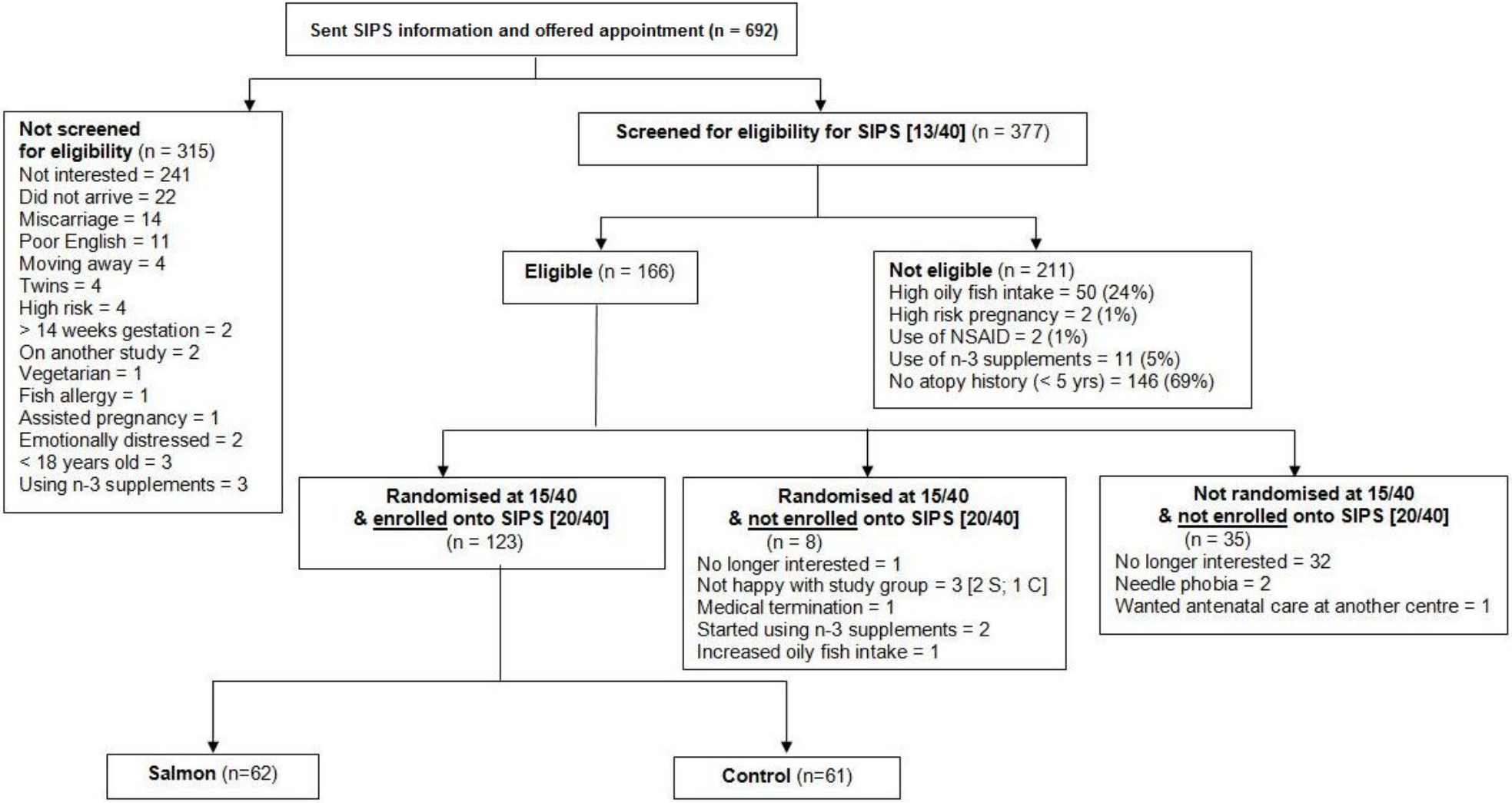
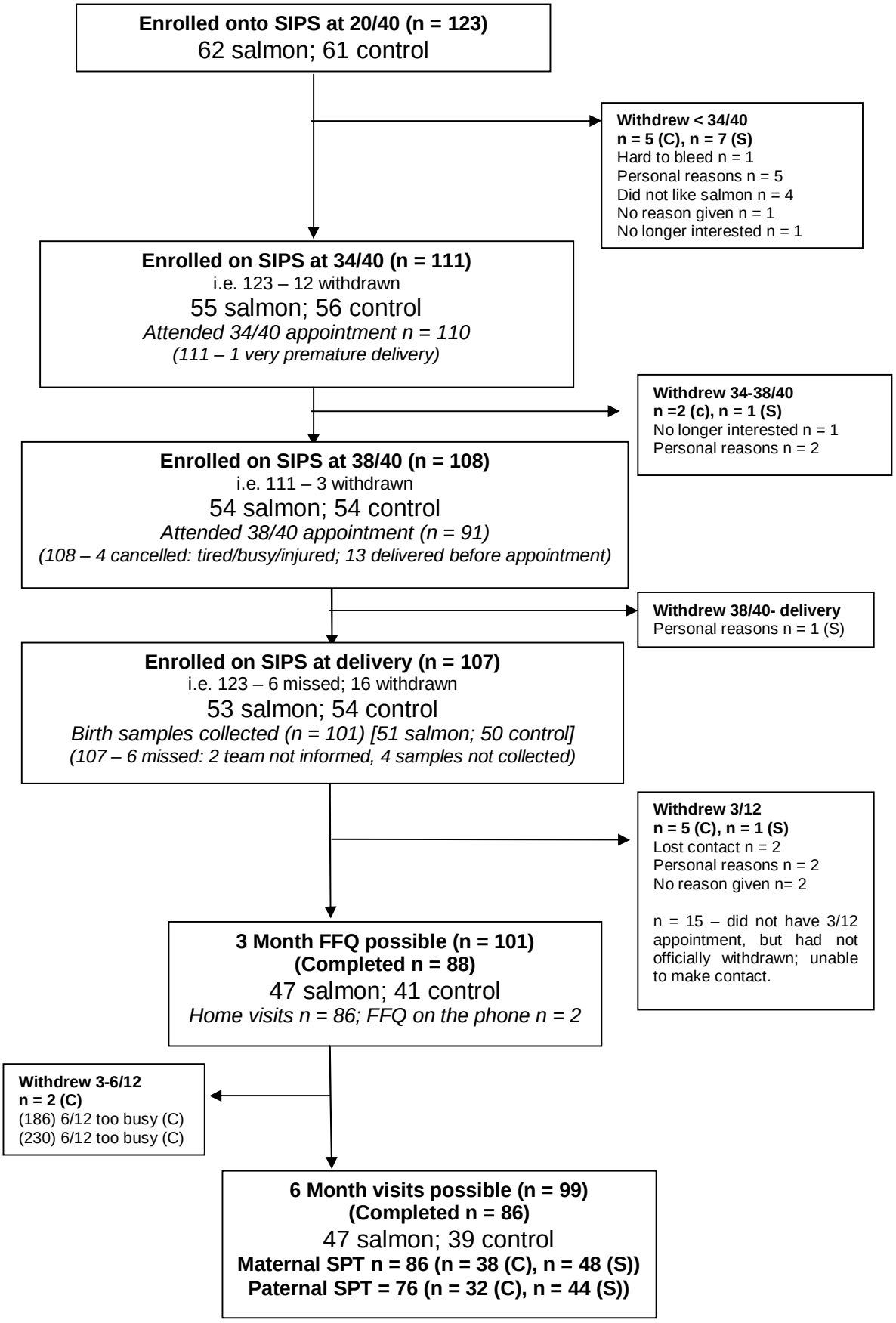


Figure 2.2 Flow of women into SIPS

Figure 2.3 Progress of the study



2.6 Subject baseline characteristics

The baseline characteristics of the subjects are presented on Table 2.7. Age, weight, height, number of pregnancies (gravida), smoking, nutritional supplement use, alcohol intake, education, and keeping pets in the home did not differ between groups. Also the groups did not differ in the frequency of conducting strenuous, moderate and gentle physical activity (Figure 2.4- Figure 2.6). However, the groups differed significantly in the number of children the women already had. More women in the salmon group had no children, and more women in the control group had 1 child ($p = 0.013$).

Table 2.8 shows the self reported atopy (any type) in the two groups. It was found that paternal self reported atopy was different between the groups with more fathers in the salmon group reporting atopy ($p = 0.015$). However when looking at different manifestations of atopy, the maternal and paternal types did not differ between the groups (Figure 2.7). More siblings of the foetus were reported to have asthma in the control group compared to the salmon group ($p = 0.11$) (Figure 2.7); however this may be related to the fact that subjects in the control group had more children as mentioned above.

Table 2.7 Baseline characteristics of SIPS subjects according to treatment group

	Control (n = 61)	Salmon (n = 62)	*p*
Maternal age (years) (13 week questionnaire)[1]	28.4 (4.7)	29.5 (4.3)	0.174
Maternal height (cm) (13 week questionnaire) [1]	165.6 (6.8)	165.4 (6.2)	0.885
Maternal weight (kg) (13 week questionnaire) [1]	71.4 (15.4)	67.4 (11.9)	0.117
Gestation length (days) [1]	279.55 (9.3)	281.8 (7.6)	0.277
Number of children (13 week questionnaire)[2]			
None	26 (42.6%)	38 (61.3%)	
1	23 (37.7%)	9 (14.5%)	**0.013**
≥ 2	12 (19.7%)	15 (24.2%)	
Pregnancies including this (13 week questionnaire)[2]			
1	23 (37.7%)	27 (43.5%)	
≥ 2	38 (62.3%)	35 (56.5%)	0.509
Maternal smoking (13 week health questionnaire) [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 supplement use (20 week FFQ) [2]			
No	15 (24.6%)	14 (22.6%)	0.966
Folic acid containing	45 (73.8%)	47 (75.8%)	
Non-folic acid containing	1 (1.6%)	1 (1.6%)	
Maternal alcohol intake (20 week health questionnaire) [2]			
No	46 (78%)	46 (76.7%)	0.866
Yes (1-7 units/week)	13 (22%)	14 (23.3%)	
Maternal education (20 week questionnaire) [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 (20 week questionnaire)[2]			
School	17 (29.8%)	16 (25.8%)	0.055
Further	25 (43.9%)	17 (27.4%)	
Higher	15 (26.3%)	29 (46.8%)	
Cat as a pet (13 week questionnaire)[2]			
No	43 (70.5%)	44 (71%)	0.954
yes	18 (29.5%)	18 (29%)	
Dog as a pet (13 week questionnaire)[2]			
No	49 (80.3%)	50 (80.6%)	0.965
Yes	12 (19.7%)	12 (19.4%)	
Other pet (13 week questionnaire)[2]			
No	42 (68.9%)	49 (79%)	0.198
Yes	19 (31.1%)	13 (21%)	

[1] Values are mean (standard deviation). Independent *t*-test *p*-values

[2] Number of subjects (within group percentage). χ^2 (chi-square) test *p*-values

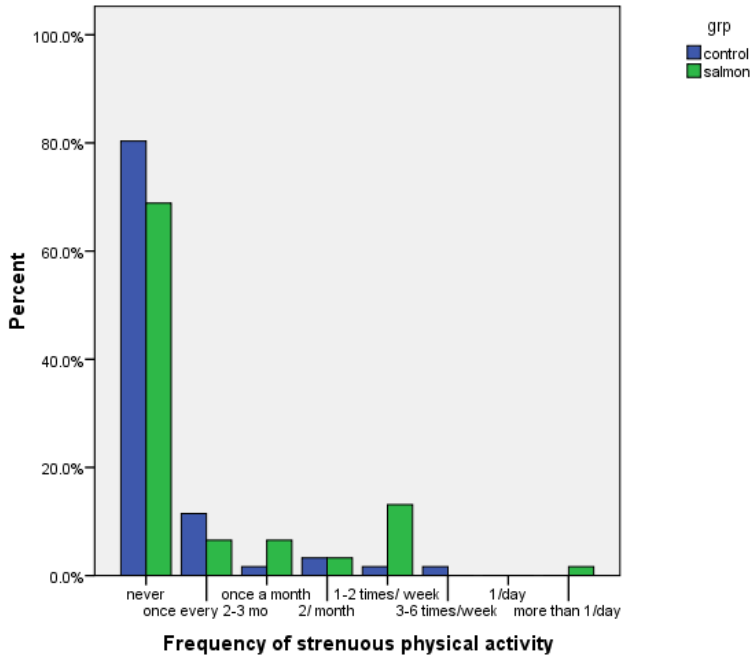


Figure 2.4 Distribution of frequency of conducting strenuous physical activity in the two treatment groups

χ^2 (chi-square) test $p = 0.102$

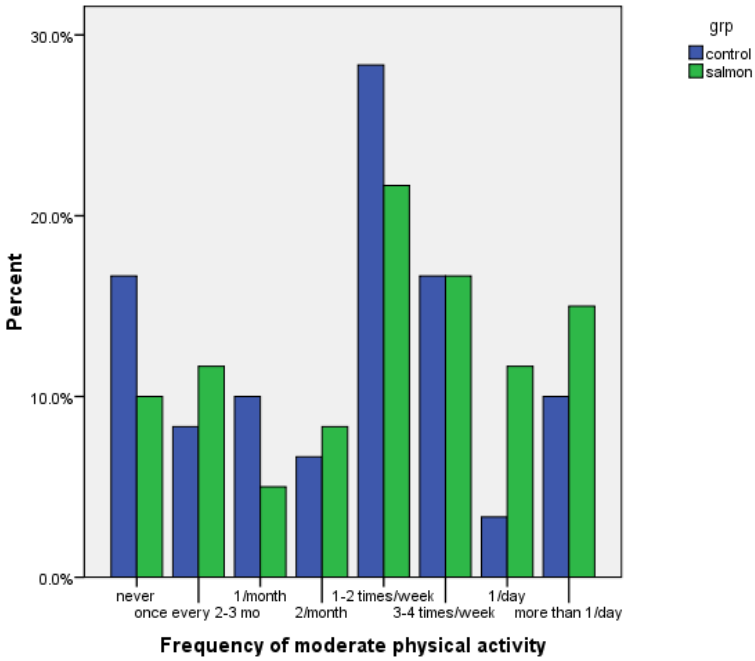


Figure 2.5 Distribution of frequency of conducting moderate physical activity in the two treatment groups

χ^2 (chi-square) test $p = 0.499$

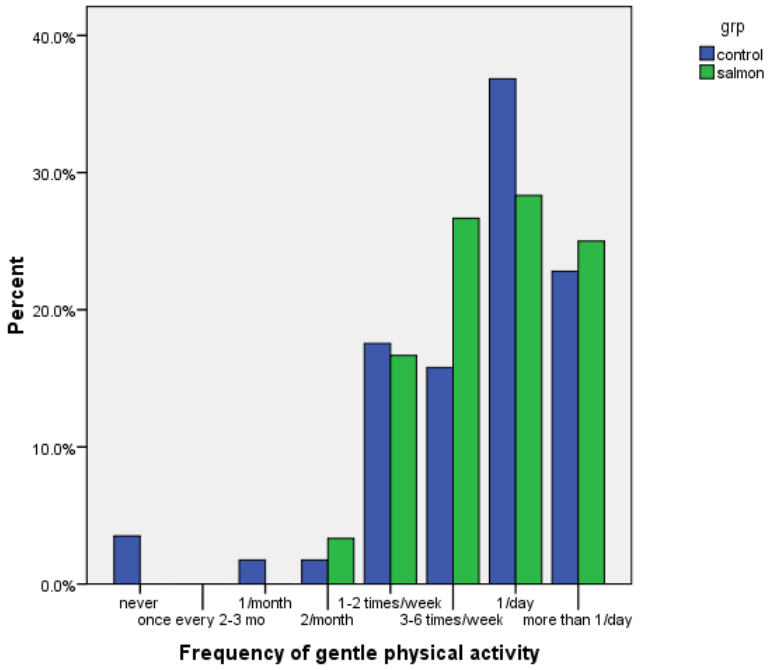


Figure 2.6 Distribution of frequency of conducting gentle physical activity in the two treatment groups
χ^2 (chi-square) test $p = 0.448$

Table 2.8 Self reported atopy at the 13 week health screening

	Control (n = 61)	Salmon (n = 62)	*p*
Maternal self reported atopy (any) [1]			
Yes	38 (62.3%)	43 (69.4%)	0.409
No	23 (37.7%)	19 (30.6%)	
Paternal self reported atopy (any) [1]			
Yes	37 (60.7%)	50 (80.6%)	**0.015**
No	24 (39.3%)	12 (19.4%)	
Sibling self reported atopy (any) [1]			
Yes	17 (27.9%)	10 (16.1%)	0.116
No	44 (72.1%)	52 (83.9%)	

[1] Number of subjects (within group percentage). χ^2 (chi-square) test *p*-values

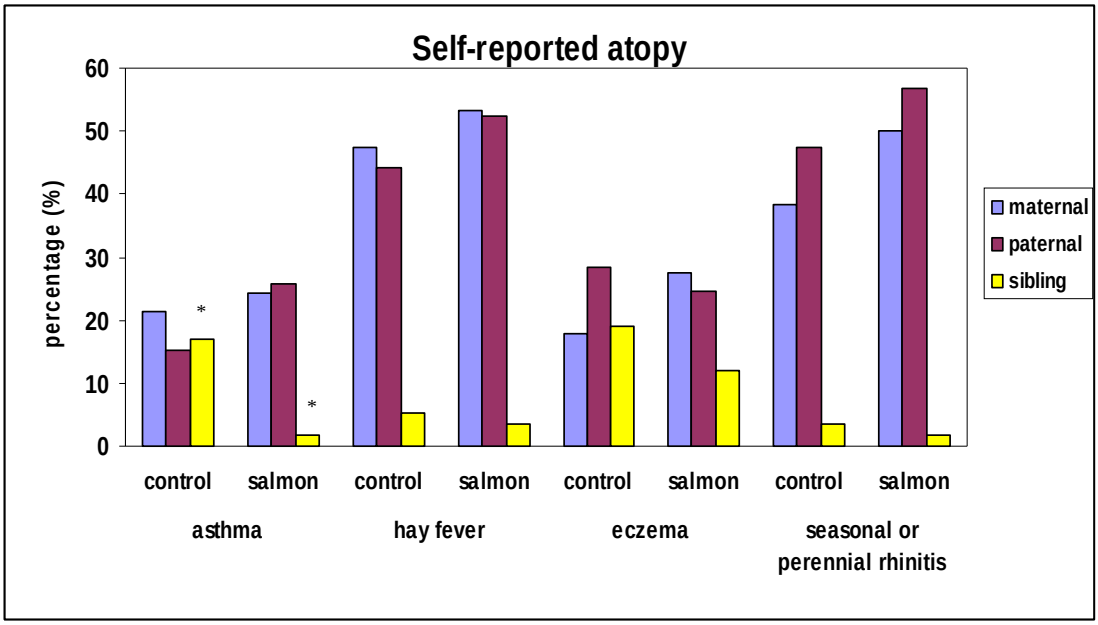


Figure 2.7 Distribution of self reported atopy categories in the two treatment groups at the 13 weeks heath screening

*χ^2 (chi-square) test $p = 0.011$

The two groups did not differ according to maternal and paternal positive skin prick testing (SPT) (Table 2.9). The number of subjects with positive SPT to specific allergens can be seen on Table 2.10. Finally, Table 2.11 shows how self reported atopy (assessed at the beginning of the study) matched with SPT (determined at 6 months postpartum) for the mothers and fathers. No statistically significant differences were found between the two groups. Within mothers who were skin-prick tested, 44.7% had reported atopy and this was confirmed by any positive SPT in the control group, and 52.1% in the salmon group. Also, within fathers who were tested, 59.4% had self reported atopy and this was confirmed by any positive SPT in the control group, and 68.2% in the salmon group. A small number of subjects who reported atopy did not have any positive SPT, and a small number of subjects were found to have positive SPT but had not reported atopy.

Table 2.9 Any positive maternal and paternal skin prick tests (SPT)

		Control	Salmon	*p*
Maternal +SPT (any) [1]		(n = 38)	(n = 48)	
	Yes	21 (55.3%)	31 (70.5%)	0.380
	No	17 (44.7%)	17 (35.4%)	
Paternal +SPT (any) [1]		(n = 32)	(n = 44)	
	Yes	20 (62.5%)	31 (70.5%)	0.466
	No	12 (37.5%)	13 (29.5%)	

[1] Number of subjects (within group percentage). χ^2 (chi-square) test *p*-values

Table 2.10 Number of subjects sensitised to each allergen assessed by skin prick testing (SPT)

Skin Prick Test Solution	Maternal - Control (n = 38)	Maternal - Salmon (n = 48)	Paternal - Control (n = 32)	Paternal - Salmon (n = 44)
Dermatophagoides pteronyssinus	14	23	13	25
Felis domesticus (cat)	11	17	6	17
Canis familiaris (dog)	12	19	11	20
6 Grass mix	18	26	16	26
3 Tree mix	6	14	5	16
Alternaria alternata	2	6	2	8
Aspergillus fumigatus	4	2	0	5
Cladosporium herbarum	0	1	1	4
Penicillum notatum	1	0	0	0

Table 2.11 Maternal and paternal self reported atopy confirmed by skin prick testing (SPT)

		Control	Salmon	*p*
Maternal [1]		(n = 38)	(n = 48)	
	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 [1]		(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%)	

[1] Number of subjects (within group percentage out of those skin-prick tested)
χ^2 (chi-square) test *p*-values

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

3.1 Introduction

Seafood, especially oily fish, (and also fish oils) are rich sources of the longer-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). In oily fish these fatty acids are found in relatively high amounts in the flesh, while in non-oily (white) fish they are found in the flesh but at lower levels and they also accumulate in the liver. Examples of oily fish are salmon, sardines, pilchards, mackerel, herring, trout, and fresh tuna. The average content of EPA plus DHA in a portion (140 g) of oily fish is between 1 and 3.5 g, depending mainly upon the type of fish (12). Typical LC n-3 PUFA contents of different types of fish and seafood see shown in (Table 1.3 transfer). The National Diet and Nutrition Survey (NDNS) (2000/2001) data showed that UK adults have a mean consumption of total fish of 217 g/week (about 1.55 portions/week), of which white fish (including fish products and dishes) was 103 g/week and oily fish (excluding tinned tuna) was 50 g/week (about 0.36 portion/week) (12). The results of the most recent NDNS (2008/2009) showed that women aged 19-64 years (including non-consumers) consume on average 7 g/day (49 g/week) coated and fried white fish, 15 g/day (105 g/week) other white fish, shellfish and tinned tuna, and 11 g/day (77 g/week) of oily fish. The same report, showed that amongst female consumers of oily fish (29% of women) aged 19-64 years old daily consumption was 39 g/day (273 g/week or 2 portions/week) (13). This data shows that over 70% of women aged 19-64 years do not consume any oily fish, although within the 30% of consumers consumption is in accordance with the minimum recommendation. The most recent NDNS data (13) did not report on intake of LC n-3 PUFA. However, the previous NDNS results (12) showed that mean intake of EPA plus DHA for adults in the UK was 244 mg/day from fish and animal sources (12). However, since about 70% of the adult population do not eat oily fish, this is an overestimate of true average intake which is likely to be 100 mg/day (14) or less (15). Also, according to NDNS (2008/2009) 2% of the population aged 19-64 years is vegetarian (13). However, it has been previously shown that the percentage of women in the UK who are vegetarians may be as high as 12% (12). Exclusion of meat and fish can result in low intake of EPA and DHA among pregnant vegetarian women. The intake of DHA among pregnant vegetarian women has been estimated around 10-30 mg/day (16, 17).

It is currently recommended to the general population to consume a minimum of 450 mg EPA plus DHA per day (11). Also, it has been recommended that DHA intake should be at least 200 mg/day in women of reproductive age (19).The Committee on Medical Aspects of Food Policy (COMA) indicates that this intake should be achieved through the

consumption of at least two portions of fish per week, of which one should be oily. This recommendation is the same for pregnant women and women of reproductive age. However, women in these groups are further advised not to consume more than two portions of oily fish and tinned tuna per week because of potential for high levels of contaminants (such as mercury). They are also advised to avoid shark, marlin and swordfish (11).

The provision of EPA and DHA to the foetus is important for foetal growth and development (11). This relates to specific roles of these fatty acids in brain and visual development (206). These fatty acids may also be important for optimal development and functioning of the vascular, cardiac, and immune systems. With regard to the latter there is some evidence that early fish and LC n-3 PUFA exposure protects against immune dysfunctions like sensitisation to allergens (atopy) and allergy and asthma in infancy and childhood (see section 1.5; (103). These effects have been linked to alterations in the foetal or infant immune system (103).

Oily fish are also good sources of nutrients other than LC n-3 PUFA, including vitamin D and selenium. Early fish and vitamin D exposure improves bone health in infancy and childhood, and it has been suggested that vitamin D supplementation in pregnant women especially during winter months may reduce the risk of osteoporotic fracture in the offspring (207, 208). Maternal vitamin D intake during pregnancy has been associated with decreased risk of sensitization to food allergens in the offspring (209) and evidence shows the importance of vitamin D in reducing risk of atopic disease development (210-212). Also, low status of antioxidants such as selenium has been found to be associated with increased risk of atopy in neonates (92, 210, 213).

Thus, a higher consumption of fish especially oily fish in pregnancy appears to be linked to better health outcomes in the offspring in infancy and childhood, and the protective effect of oily fish is linked to the nutrients that it contains particularly LC n-3 PUFA, vitamin D and selenium. Protective effects of the nutrients have been or are being examined in intervention studies (22, 92, 183, 209-213). However, there are no published intervention trials with fish during pregnancy. Furthermore, there have been no studies so far implementing an intervention on a population level which aims to sustainably alter fish intake, and thus the factors affecting sustainability of change of fish intake have not been examined. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish (in this case farmed salmon) in pregnant women. SIPS specifically focuses on pregnant women whose offspring are at high risk of developing atopic disease, with one

aim being to identify whether there is an effect on atopy outcomes in the offspring. As described in chapter 2, a total of 123 pregnant women were randomly assigned to either consume 2 portions of farmed salmon per week from week 20 of pregnancy until delivery or to continue their habitual diet low in oily fish (less or equal to 2 portions per month). The aim of the work described in this chapter is to characterise the habitual diets and nutrient intakes of the women enrolled in SIPS, to identify whether diet changes over the course of pregnancy in this group, to examine compliance to the SIPS intervention, and to identify the effect of the intervention on maternal nutrient intake.

The primary hypotheses examined by the work described in this chapter are that:

- the salmon intervention will increase oily fish consumption from a habitually low intake (≤ 2 portions per month) to the recommended maximum level of intake, which is 2 portions per week during pregnancy;
- the salmon intervention will result in increased maternal LC n-3 PUFA intake during pregnancy, and that this intake will be higher than in the control group.

The secondary hypothesis examined by the work described in this chapter are that:

- the salmon intervention will result in changes in dietary patterns for the salmon group, through the replacement of other foods with the study salmon, such as meat and other types of fish;
- the salmon intervention will result in a sustained increase in oily fish intake after the end of the intervention (post-partum);
- the salmon intervention will increase intake of other nutrients provided by the study salmon, such as selenium and vitamin D and that intake of these nutrients will be higher than in the control group.

3.2 Methods

3.2.1 Study design, subjects, subject characteristics and the intervention

In chapter 2 the study design, the recruiting procedure, the nature of the women recruited and their characteristics, and the nature of the intervention were all described in detail. One group of pregnant women (n = 61) did not undergo any dietary intervention. A second group of pregnant women (n = 62) consumed two portions of farmed salmon per week from week 20 of pregnancy until delivery. Full details of the salmon are given in section 2.4. They provided 1.73 g EPA plus DHA per portion (i.e. 3.46 g per week), 14 µg vitamin D per portion (28 µg per week) and 43 µg selenium per portion (86 µg per week). NDNS data suggests that average consumption of these nutrients among young adult women is < 200 mg/day (or < 1.4 g/week) for EPA plus DHA (12), 2.7 µg/day (or 18.9 µg/week) for vitamin D and 39 µg/day (or 273 µg/week) for selenium (13). Women completed a food frequency questionnaire (FFQ; see below) at weeks 20 and 34 of pregnancy and again at 3 months post-partum. They completed diaries recording fish intake from week 19/20 of pregnancy until delivery.

3.2.2 Assessment of fish, food and nutrient intakes

3.2.2.1 General considerations

An important issue with dietary interventions is compliance to the intervention and the method by which compliance is measured and controlled. Although weighed food diaries are considered to be more accurate when determining nutrient intake for a small number of volunteers, compared to FFQ, they cannot reflect a long period of time because of the difficulties associated with long-term completion by volunteers. They typically cover a 4 to 7 day period. On the other hand, a well designed FFQ can reflect the dietary patterns as well as the nutrient intake over a longer period of time, especially for large populations (103, 214). Although the SIPS sample size was small, because of the primary interest in fish intake and the long period of intervention, it seemed appropriate to use a FFQ especially developed for use in the Southampton population. The FFQ used was developed for use in the Southampton Women's Survey (SWS); this FFQ included questions on consumption frequency of different types of fish and reflected diet over the previous three months. In addition, in order to assess more closely compliance of both salmon and control groups, volunteers completed a specific record of fish consumption, termed a 'fish diary'. The combination of the two methods allows control over compliance, and also provides the

information required to investigate dietary patterns and nutrient intake and changes in these. The SWS FFQ has been validated for use to assess nutrient intakes in pregnant women in Southampton (214-217). The SWS is a study of a population sample of non-pregnant women aged 20 to 34 years in Southampton. Women who subsequently become pregnant were followed up. Complete dietary data are available for 12,572 non-pregnant women, 2,270 women in early pregnancy and 2,649 women in late pregnancy (218). Details on the SWS have been published previously (201). Robinson *et al.* (217) compared early pregnancy nutrient intakes assessed by FFQ with those determined from food diaries kept over four days. It was concluded that the FFQ can give meaningful estimates of nutrient intake in early pregnancy. Godfrey *et al.* (219) used the same FFQ in early and late pregnancy to assess the relationship between maternal nutrient intake and placental and foetal growth. The description of FFQ and fish diaries can be found in the following section of this chapter.

3.2.2.2 The food frequency questionnaire

Diet was assessed using a nutritionist-administered (L.-S. Kremmyda and M. Vlachava) 100-item FFQ covering food intake over the preceding twelve weeks (Appendix H). The nutritionists administering the FFQ were blinded to the treatment group of the subjects. The FFQ was administered at 20 and 34 weeks gestation, reflecting dietary intake before (8 to 20 weeks gestation) and during intervention (22 to 34 week gestation). Moreover, the FFQ was administered 3 moths after delivery to assess dietary intake after intervention, reflecting the first three months post-partum.

The FFQ reflected intake of various 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 other food items, the FFQ included questions of consumption frequency of different types of fish: ‘oily fish’, ‘non-oily fish’ (including tinned tuna), ‘fish fingers and fish dishes’, and ‘shellfish’. Also, volunteers were asked to think about composite food dishes, including foods they ate out or cooked themselves. The FFQ also included questions on nutritional supplement/vitamin/mineral intake and alcohol intake. Finally, for the purposes of SIPS, a question on functional food products with added n-3 and/or n-6 fatty acids, as well as probiotic and prebiotic products, was added at the end of the FFQ. Subjects were asked to indicate the brand, quantity and weekly frequency of any functional food product they had consumed on a regular weekly basis in the past three

months. Prompt cards with an extensive list of such products in the market were used to help the volunteers with answering.

Subjects were asked to recall their intake during the preceding three months, and to ensure standardised responses prompt cards with examples of food items included in each food category were used. Categories of consumption frequency were also presented on a prompt card and they 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'. Weekly consumption frequencies for selected food items were calculated. As for most FFQ, this questionnaire had an ordinal scale of distinct consumption frequency categories, rather than an interval scale providing a 'true' measure (220). Therefore, the above frequency categories were transformed to the following weekly frequencies: 'times per day multiplied by seven', '7 times per week', '4.5 times per week', '1.5 times per week', '0.5 times per week', '0.25 times per week', '0.1 times per week', '0 times per week'. The weekly frequencies were used as multiplication factors to calculate nutrients as follows.

Standard portion sizes were assigned for each food item on the FFQ, derived from a published list for the UK (221). The nutrient content of a standard portion of each food was multiplied by its reported frequency (translated to a weekly level) to calculated weekly nutrient intake. Daily nutrient intake values were calculated after dividing weekly intake by seven (8-10). Nutrients from each FFQ food item were added up to obtain total diet intake for each nutrient. According to SWS (personal communication with Robinson SM, MRC SWS nutritionist), nutrients declared to have complete values and to provide a reliable estimate of intake from the total diet are the following: energy, carbohydrates, protein, total fat, saturated fat (SFA), monounsaturated fat (MUFA), polyunsaturated fat (PUFA), total n-3 PUFA, total n-6 PUFA, dietary fibre, sodium, potassium, calcium, magnesium, phosphorus, iron, selenium, zinc, retinol, carotene, thiamin, riboflavin, niacin, vitamin B6, vitamin B12, folate, vitamin C, vitamin E, vitamin D. Selenium values are complete, however, they were used with caution as selenium content varies widely and depends on soil content (222). As a result total dietary selenium intake was treated as a rough estimate.

3.2.2.3 Dietary patterns throughout the study

In order to determine possible changes occurring in the diets of the pregnant women in relation to the intervention and/or to pregnancy, FFQ food items were grouped. All 98 foods and non-alcoholic drinks of the FFQ were combined to form 48 food groups. Each food group included foods of similar nutrient composition and comparable usage,

according to the food grouping performed by Crozier *et al.* (218) for the SWS FFQ. For instance, salad, coleslaw, tomatoes, green peppers and watercress formed the 'salad vegetables' group; white fish, fish fingers and fish dishes, oily fish, and shellfish were combined into the 'total fish and shellfish' group. Table 3.1 presents the way all food items were grouped. Weekly consumption frequency of these food groups was calculated by summing the weekly consumption frequency of each food item.

Table 3.1 Food frequency questionnaire food grouping

Food group created	Foods included in each group
Rice and pasta	Rice, pasta
White bread	White bread
Wholemeal bread	Wholemeal bread
Quiche and pizza	Quiche and pizza
Savoury pancakes (including Yorkshire pudding)	Yorkshire pudding and savoury pancakes
Breakfast cereals	'Bran' breakfast cereals, 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 (pints)	Full-fat liquid milk
Reduced-fat milk (pints)	Reduced fat liquid milk
Yogurt	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
Processed meat	Bacon and gammon, meat pies, sausages, ham and canned meats
Total fish and shellfish	White fish, fish fingers and fish dishes, oily fish, shellfish
Salad vegetables	Salad, coleslaw, tomatoes, green peppers and 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 confectionary
High-energy soft drinks	Blackcurrants and blackcurrant drinks (Ribena), fizzy drinks and squashes, cola drinks
Diet cola	Diet cola drinks
Tea and coffee	Tea, coffee
decaffeinated tea and coffee	Decaffeinated tea (including herbal) and decaffeinated coffee
Hot chocolate drinks	Drinking hot chocolate and milkshakes
Miscellaneous	Soup, stock cubes and marmite, mayonnaise and salad cream, pickles, chutney and ketchup, added bran to foods

Principal component analysis (PCA) was used to assess dietary patterns based on the 48 food groups formed. PCA is a multivariate statistical method that has been used by a number of studies to assess changes or stability of dietary patterns (223). It produces new variables that are uncorrelated linear combinations of the dietary variables with maximum variance (224). The PCA was based on the correlation matrix to adjust for unequal variances of the original variables. PCA generates coefficients for each of the food groups in the analysis, and a dietary pattern score for each individual is calculated by multiplying these coefficients by the individual's standardised reported consumption frequencies. When multiplying the coefficients derived from PCA at one time point by each individual's standardised reported frequencies of consumption at that time point [reported consumption frequency-mean consumption frequency)/standard deviation of consumption frequency], the dietary pattern scores produced are termed as 'natural' scores (218). On the other hand, when dietary pattern scores are calculated using coefficients derived from PCA performed on a different time point they are termed as 'applied' scores (225). Applied scores have been used by various studies to examine the change in individual pattern scores over time. In these studies, scores at follow-up time points are calculated based on coefficients derived by PCA at baseline (215, 226). In a follow-up setting, when

calculating applied dietary pattern scores, the coefficients for each food group obtained from the PCA at baseline are multiplied by the frequencies of consumption at the follow-up time point standardised to the mean and standard deviation observed at baseline. An advantage of applied scores is that the scale of measurement (the dietary pattern) remains constant. Applied scores are generally preferred to natural ones (218). The SWS PCA revealed a first principal component that was characterized by high intakes of vegetables (peppers, tomatoes, vegetable dishes, courgettes, green salad, onions and spinach), wholemeal bread, vegetarian food and pasta, and low intakes of full-fat milk, beef, crisps and savoury snacks, Yorkshire pudding and savoury pancakes, white bread, sugar, gravy, sausages, meat pies and roast potatoes. This first component was termed 'prudent' diet score in accordance with published data (218, 227). Women with higher 'prudent' diet scores had diets in accordance with recommendations from the Department of Health (UK) (228).

Using the SIPS FFQ data, PCA was conducted and coefficients derived for the 48 food groups at each time point at which the FFQ was administered (i.e. 20 and 34 weeks gestation, and 3 months post-partum). Further, the SIPS coefficients were correlated with the SWS ones (before pregnancy coefficients) (218). Figure 3.1 shows that SIPS coefficients (20 week time point) correlated very well with SWS coefficients: Pearson's correlations were 0.853 at 20 weeks gestation, 0.868 at 34 weeks gestation, and 0.635 for 3 months postpartum (all $p<0.01$). On the basis that the SIPS study was conducted in Southampton, the SIPS cohort should have similar dietary patterns to the SWS, although the small sample size of SIPS is potentially a limitation in calculating food group coefficients from PCA. Based on this, the SWS coefficients were applied to the SIPS consumption frequency data in order to calculate applied dietary pattern scores for each individual (Southampton Women's Survey/ Salmon in Pregnancy Study (SWSIPS) 'prudent' score). SWS coefficients for each food group were multiplied by each individual's reported consumption frequencies standardised to the mean and standard deviation observed in SWS (because standardising to the frequencies at each time point would remove information about changes in consumption between time points). The applied dietary pattern scores were standardised to the mean and standard deviation of the score [SWSIPS 'prudent' z-score = (individual SWSIPS 'prudent' – mean SWSIPS 'prudent')/ standard deviation of SWSIPS 'prudent'], so that the units of the scores were meaningful (standard deviations).

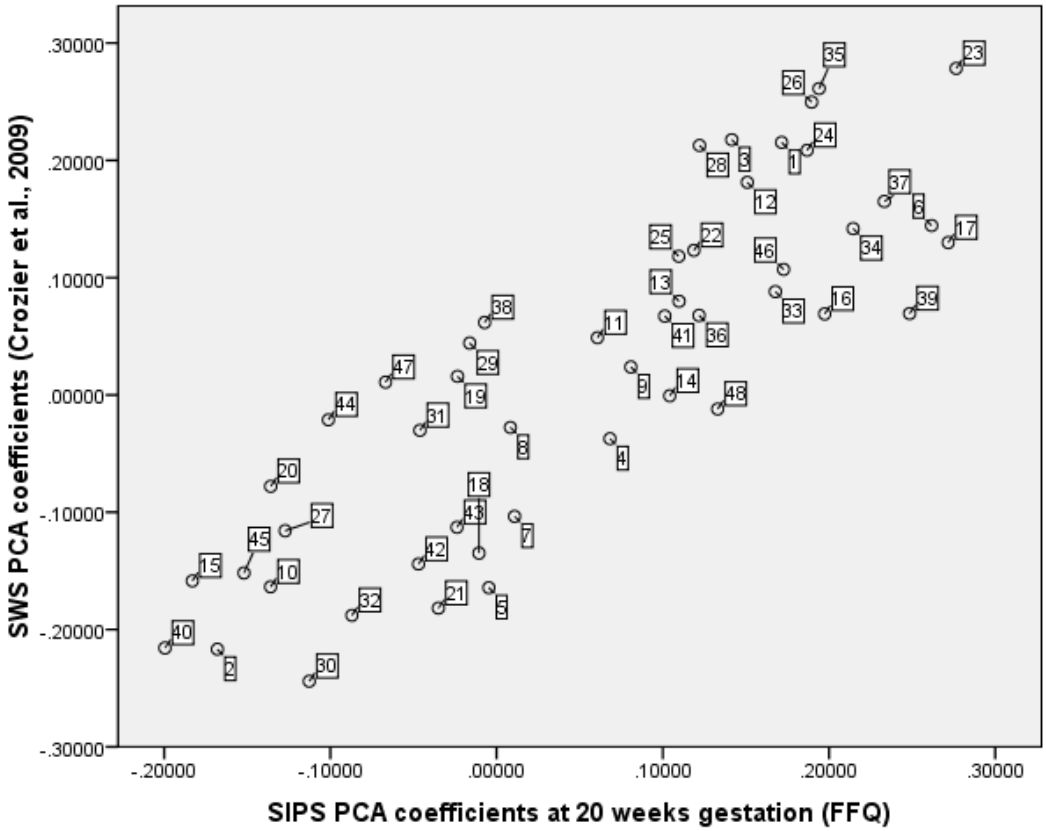


Figure 3.1 Correlation between SWS and SIPS (at 20 weeks gestation) PCA coefficients
Pearson's correlation 0.853, $p < 0.01$

Additional food groups created which were not included in PCA were: total fruit, total meat, total vegetables, and total alcohol. These food groups were created in the same way as for the 48 food groups for PCA, summing up the consumption frequencies of individual FFQ food items or food groups. For example, total fruit was created by the combination of all fruit and fruit juice categories from the 48 PCA food groups. Moreover, the following 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): margarine, butter, olive oil based spreading fat, vegetable oils, olive oil, animal fat, and salad dressings.

3.2.2.4 Fish diaries

All volunteers were asked to keep a 'fish diary'. This was a diary in which they were asked to fill in the quantity, exact name, and cooking method of any fish consumed during

pregnancy. For those who were in the salmon group, they were also asked to fill in exactly when they consumed the study salmon, how much, and how it was cooked. The fish diary had the form of a calendar including all days and weeks of pregnancy, between the start of the intervention (20 weeks gestation) and delivery. The fish diaries were used as a measure of compliance during intervention for both groups, in addition to the FFQ, and to estimate fish consumption frequencies and nutrient intakes from fish. The fish consumed were grouped into the following fish categories, to match the FFQ fish categories: 'study salmon', 'oily fish' (including study salmon and other oily fish), 'non-oily fish', 'fish fingers and fish dishes', shellfish', and 'total fish'. Weekly consumption frequencies were multiplied by fish nutrient contents (see below) to calculate weekly nutrient intake, and divided by seven to obtain daily nutrient intakes. Mean weekly consumption frequency of different fish categories and mean daily nutrient intakes were calculated only for the period 21 to 38 weeks of pregnancy. Week 39 of gestation to delivery was not included in the analysis as the women were very close to delivery and diary keeping was generally poor during that period.

3.2.2.5 Fish nutrient content

Due to the fact that the study salmon was tailor-made and chemically analysed (see section 2.4), the contents of seven nutrients of interest were calculated accurately. This information, along with information from other nutrient databases (8-10), was used to update the fish nutrient content for all of the fish categories as SIPS had a particular interest in fish consumption. The fish recipes were chosen by the SIPS nutritionists (L-S Kremmyda and M Vlachava) to match with the fish habitually consumed by the volunteers in the study. This was based on the information given by the fish diaries which included the exact type of fish consumed. Table 3.2 provides information on which fish were used to create the fish recipes in each fish and seafood category, and Table 3.3 shows content of the seven nutrients of interest according to these recipes. The nutrients per portion were multiplied, as described above, by the weekly consumption frequency given by the FFQ and fish diaries to calculate weekly intake of the eight nutrients of interest from fish. Daily intakes were obtained after dividing by seven. These fish recipes were also incorporated when calculating total diet nutrient intake.

Table 3.2 Fish used to obtain nutrient content of each FFQ fish category

Fish category	Individual fish in each category
White fish	Cod, haddock, plaice, tinned tuna in water (drained), tinned tuna in oil (drained)
Oily fish	Salmon pink/red tinned in brine, mackerel, trout
Shellfish and seafood	Prawns, mussels, scampi, crab
Fish fingers and dished	Fish cakes, fish fingers

All portions were set at 150 g. Equal amounts of each individual fish/seafood were used to create the fish/ seafood group

Table 3.3 Nutrient content in a standard portion (150 g) of each fish/ seafood category

Fish category	Total n-3 PUFA (g)	Total n-6 PUFA (g)	EPA (mg)	DHA (mg)	Se (µg)	Vit D (µg)	Vit E (mg)
White fish	0.642	1.008	111	231	78	1.98	1.245
Oily fish	2.975	0.595	740	1355	38	13.5	1.48
Study salmon	3.56	2.31	570	1160	43	14	4.1
Shellfish and seafood	0.366	0.092	176	122	31	tr.	78.7
Fish fingers and fish dishes	0.25	0.94	45	60	21	tr.	na.

tr., trace; na., value not available

3.2.3 Statistical analysis

FFQ. Between group (salmon-control) comparisons for 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: food group consumption frequency, meat and fish consumption frequency, nutrients from white, oily, and total fish, and specific nutrient intake from total diet. Between group comparisons for normally distributed data were performed using the independent samples t-test, and the data are described using means and standard deviations (sd): specific nutrient intake from total diet, SWSIPS 'prudent' diet z-score, changes in meat and fish consumption frequencies (34 minus 20 weeks gestation, and 3 months postpartum minus 20 weeks gestation), changes in nutrient intake from fish (34 minus 20 weeks gestation). Functional food consumption was categorised in the following consumption frequencies: none, $\geq$ 3 portions/week, $\leq$ 4 portions/week, and the Pearson χ^2 (chi-square) test was used to identify differences between the groups. Generalised linear modelling was used to perform a 3 repeated measures analysis of variance (ANOVA). This was used to examine overtime changes during pregnancy (20 and 34 weeks gestation) and 3 months post-partum in relation to the intervention: meat and fish consumption frequencies, nutrient intake from total fish.

Fish diaries. Between group comparisons for 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: mean weekly consumption frequency of fish categories, mean daily nutrient intake form white, oily, and total fish (period 21 to 38 weeks gestation).

A *p*-value of 0.05 was considered statistically significant for all tests. The Statistical Package for Social Sciences (SPSS) version 16 was used for all statistical tests. SIPS PCA coefficients were calculated using Stata (version 10).

3.3 Results

3.3.1 Food groups consumption frequencies based upon FFQ

Consumption frequencies of the 48 food groups, according to the SWS categorisation for PCA, for all time points of FFQ administration and for both groups, are described in Table 3.4. Although the volunteers were randomly assigned into the two groups, consumption frequencies at baseline (20 week gestation FFQ reflecting the preceding 3 months) differed significantly between control and salmon group for the following food groups: whole-meal bread, total fish and shellfish, other fruit juices, sweets and chocolate. The salmon group had higher intake for all 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 with that of the control group which was 0.75 times per week at baseline (20 weeks gestation FFQ) ($p < 0.001$). The proportion of women with a baseline intake of 0.5 times of oily fish per week (i.e. volunteers who just about fulfilled the criterion of 2 portions per month maximum) was 34% for the salmon group and 8% for the control group. These differences between the two groups indicate a 'healthier' dietary pattern for the salmon group compared to control at baseline, although volunteers were randomly assigned to the two groups. During intervention (34 weeks gestation FFQ), 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 statistically significant lower consumption frequency of red meat than the control group (1.75 vs. 2.5, $p = 0.022$). The result of paired tests (non-parametric Wilcoxon's Sign Rank test) showed that within the salmon group, chicken intake was significantly reduced from 20 to 34 weeks gestation ($p = 0.018$), and so was the intake of non-oily fish ($p = 0.014$) (data not shown on tables). Also, consumption frequencies of chips and roast potatoes, and sugar differed between the two groups at 34 weeks of pregnancy (lower in the salmon group). After intervention, according to the results of the 3 month post-partum FFQ, the salmon group continued to have a significantly higher consumption frequency of total fish and shellfish. There were a few other differences between the two groups at that time point.

Table 3.5 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, total meat consumption decreased significantly from 20 to 34 weeks gestation within the salmon group (Wilcoxon's test, $p = 0.048$) (data not shown on tables). The same table includes information on the different types of spreading fat, cooking fat, and salad

dressings. The only statistically significant differences between the two groups were in olive oil at 34 weeks gestation and in olive oil based spreading fat at 3 months post-partum.

Table 3.4 Between group comparisons of weekly consumption frequency of food groups in the FFQ administered at 20 and 34 weeks gestation, and 3 months after delivery

Food group	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 54)	Salmon (n = 55)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
Rice and pasta	3 (1.88, 3)	3 (1, 3)	0.533	2 (1, 3)	2 (1, 3)	0.729	3 (1.75, 3)	2 (1.6, 3)	0.371
White bread	9 (2.25, 14)	3.75 (0.94, 14)	0.096	9 (3, 14)	3 (1, 14)	0.061	9 (1, 14)	6.75 (1, 14)	0.936
Whole-meal bread	3 (0.3, 9)	4.5 (0.69, 14)	**0.048**	1.25 (0, 9)	3 (0.2, 14)	0.051	3 (0.05, 9)	3 (0.62, 9)	0.103
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	0.5 (0.25, 0.5)	0.5 (0.25, 1.5)	0.156
Savoury pancakes (incl. Yorkshire pudding)	0.25 (0, 0.5)	0.1 (0.08, 0.25)	0.298	0.1 (0, 0.25)	0.1 (0, 0.25)	0.287	0.1 (0, 0.25)	0.1 (0, 0.25)	0.496
Breakfast cereals	4.5 (0.5, 7)	4.5 (0.88, 7)	0.212	4.55 (1.5, 7)	7 (1.5, 7)	0.233	6 (1.5, 7)	4.5 (1.5, 7)	0.236
Cakes and biscuits	5.5 (3, 9.75)	4.38 (2, 9.36)	0.331	5.75 (3, 10)	8 (3.50, 11)	0.252	5 (2.75, 9.55)	5.35 (2.2, 10.5)	0.900
Puddings	1 (0.5, 2.5)	1.55 (6, 2.25)	0.691	1.68 (0.81, 2.56)	1.50 (0.75, 2.25)	0.634	1.1 (0.65, 2)	1.25 (0.7, 2.2)	0.710
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	0.25 (0, 0.5)	0.25 (0, 0.5)	0.790
Full-fat milk (pt/day)	0 (0, 0)	0 (0, 0)	0.706	0 (0, 0)	0 (0, 0)	0.851	0 (0, 0)	0 (0, 0)	0.849
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	0.5 (0.25, 0.75)	0.5 (0.25, 0.75)	0.936
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	1.5 (1, 4.5)	1.5 (2.5, 4.5)	0.208
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	4.5 (1.5, 4.5)	4.5 (1.5, 4.5)	0.740
Eggs and egg dishes	0.5 (0.10, 1.75)	0.5 (0.25, 1.13)	1.000	0.5 (0.2, 1.5)	0.5 (0.2, 1)	0.736	0.75 (0.25, 1.6)	0.5 (0.25, 1)	0.218
Full-fat spread	4.5 (0, 7)	1.5 (0, 7.38)	0.712	4.5 (0, 7.38)	4.5 (0.25, 9)	0.701	4.5 (0, 7)	4.5 (0, 7)	0.769
Reduced-fat spread	0 (0, 5.25)	1.5 (0, 7)	0.108	0 (0, 7)	0 (0, 7)	0.979	0 (0, 4.55)	0 (0, 7)	0.822
Cooking fats and salad oils	3 (1.5, 4.5)	3.13 (1.5, 6)	0.234	3 (1.5, 5.06)	4.5 (1.5, 6)	0.348	3 (1.5, 5)	4.5 (1.85, 7)	0.143
Red meat	2.5 (1, 3.5)	2.05 (0.95, 3.1)	0.354	2.5 (1.75, 3.53)	1.75 (0.95, 3)	**0.022**	2.75 (1.7, 3.68)	2 (1, 3.5)	0.163
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	1.5 (1, 1.5)	1.5 (5, 1.5)	0.619
Liver, liver pate, and kidney	0 (0, 0)	0 (0, 0)	0.545	0 (0, 0)	0 (0, 0)	0.394	0 (0, 0.15)	0 (0, 01)	0.883
Processed meat	2.75 (1.43, 5.3)	2.75 (1.83, 4.81)	0.877	2.43 (1.18, 3.64)	2.5 (1.25, 5.1)	0.501	3 (2, 4)	2.5 (1.5, 4)	0.639
Total fish and shellfish	0.75 (0.35, 2)	1.75 (0.75, 2.25)	**0.001**	0.75 (0.26, 1.85)	2.35 (2, 3)	**<0.001**	0.75 (0.18, 1.75)	1.2 (0.6, 2.5)	**0.035**
Salad vegetables	4.6 (3, 9.38)	6.88 (3.75, 10.13)	0.072	4.75 (2.71, 7)	5.25 (3.25, 9.6)	0.376	3.5 (2.5, 6.68)	6.6 (3, 9)	0.087
Green vegetables	3.7 (2.05, 5.63)	3.73 (2.1, 6.05)	0.581	3.68 (1.69, 5.31)	3.75 (2.6, 6)	0.310	5 (2, 7.25)	3.7 (2.25, 6.5)	0.716
Root vegetables	1.75 (1.5, 4.5)	1.75 (1.5, 4.5)	0.711	1.75 (1.38, 3)	1.75 (1.5, 3)	0.710	3 (1.5, 4.68)	1.7 (1, 3)	**0.023**
Other vegetables	3.5 (2, 6.5)	4.5 (2.25, 6.69)	0.966	3.25 (2.19, 6.25)	3.25 (2, 6.25)	0.956	3.5 (2.38, 6.38)	4.5 (2, 6.1)	0.630
Tinned vegetables	0.25 (0, 1.5)	0.1 (0, 0.5)	0.192	0.18 (0, 1.5)	0 (0, 0.5)	0.189	0.1 (0, 1)	0 (0, 0.5)	0.136

Food group	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 54)	Salmon (n = 55)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
Vegetable dishes	0.25 (0, 1.25)	0.25 (0, 0.94)	0.667	0.25 (0, 0.5)	0.25 (0, 0.5)	0.865	0.1 (0, 1.5)	0.25 (0, 1.5)	0.647
Beans and pulses	1.5 (0.25, 1.5)	1.5 (0.44, 1.5)	0.910	1 (0.25, 1.5)	1.5 (0.5, 1.5)	0.641	0.5 (0.5, 1.5)	1.5 (0.5, 1.5)	0.682
Chips and roast potatoes	3 (1.50, 6)	3 (1, 4.5)	0.314	3 (1.5, 4.5)	1.5 (1, 4.5)	**0.021**	3 (1.5, 4.5)	1.5 (1, 4.5)	**0.046**
Boiled potatoes	9 (4.13, 13.5)	4.5 (4.5, 6.75)	0.090	4.5 (3.56, 13.5)	4.5 (4.5, 13.5)	0.660	9 (4.5, 14.63)	4.5 (3, 13.5)	**0.046**
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	1.5 (0.5, 4.5)	1.5 (0.5, 1.5)	0.685
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	0.1 (0, 0.25)	0.2 (0, 1.5)	0.168
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	1.5 (0.5, 4.5)	4.5 (0.75, 5)	0.076
Other fruit	9.25 (5.38, 16.25)	9 (3.79, 14.38)	0.487	7.63 (3.29, 14)	8.75 (5, 14)	0.346	5.1 (2, 9.8)	6 (2.75, 10.5)	0.569
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	0.5 (0.1, 1.5)	1.5 (0.1, 4.5)	0.477
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	0.25 (0, 1)	0.1 (0, 1.5)	0.986
Cooked and tinned fruit	0.2 (0, 0.55)	0.15 (0, 0.5)	0.476	0.25 (0, 0.5)	0.25 (0, 0.5)	0.522	0.25 (0.1, 0.5)	0.2 (0, 0.5)	0.469
Nuts	0 (0, 0.25)	0.1 (0, 0.5)	0.060	0.05 (0, 0.5)	0 (0, 0.5)	0.559	0.25 (0, 1)	0 (0, 0.25)	0.095
Sugar (tsp/day)	0 (0, 3)	0 (0, 2)	0.886	1 (0, 3)	0 (0, 1)	**0.049**	0 (0, 3)	0 (0, 2)	0.489
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	0.25 (0, 1.5)	1.5 (0.5, 4.5)	**0.002**
Sweets and chocolate	5 (2, 9)	3 (1.75, 6)	**0.037**	6 (2.75, 8.63)	4.75 (2, 7)	0.216	3 (1.5, 7)	3 (1.6, 6)	0.600
High-energy soft drinks	6 (1.5, 10)	4.5 (0.50, 9.75)	0.354	7.38 (1.6, 14.03)	3 (0.5, 10.5)	0.058	3 (0.5, 7.38)	3 (0.25, 7.5)	0.873
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	0 (0, 0.5)	0.25 (0, 0.5)	0.377
Tea and coffee	11.5 (0.63, 21)	7 (1.5, 15.5)	0.508	8.75 (4.5, 21)	7 (1.5, 15.5)	0.187	14 (1.75, 24.5)	14 (4.75, 21.1)	0.763
decaffeinated tea and coffee	0 (0, 4.5)	0 (0, 5.13)	0.864	0 (0, 2.25)	0 (0, 4.5)	0.559	0 (0, 4.5)	0 (0, 4.5)	0.689
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	0.1 (0, 0.5)	0.1 (0, 0.5)	0.720
Miscellaneous	5.85 (3.05, 9)	4.75 (3.25, 8.63)	0.792	5 (3.25, 8)	5 (2.5, 8.6)	0.450	5 (2.3, 7.63)	5 (2.35, 7.6)	0.940

*_p_-values from non-parametric 2 independent samples test (Mann Whitney)
Values are median (interquartile range)
Number of times per week (apart from milk (pints/day) and sugar (teaspoons/day))
Food grouping performed according to principal component analysis (PCA) of the SWS FFQ

Table 3.5 Between group comparisons of weekly consumption frequency of additional food groups in the FFQ

Food group	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 54)	Salmon (n = 55)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
Total fruit	15.5 (10.18, 23.8)	18.88 (10.93, 28.23)	0.268	11.75 (8.4, 24.45)	18.1 (10.5, 27.1)	0.096	11 (6.6, 15.77)	12.8 (9, 20.25)	0.124
Total vegetables	18.6 (14.33, 28.3)	21.18 (15.28, 27.8)	0.491	16.8 (12.99, 26.5)	19.25 (13.7, 25.5)	0.476	20.25 (14.77, 23.85)	18.25 (13.2, 26.5)	0.664
Total meat	7.35 (5.1, 11.68)	7.3 (5.49, 10.2)	0.638	7.25 (4.43, 10.64)	6.45 (4.55, 9.5)	0.495	7.8 (5.6, 10.02)	7 (5.5, 8.75)	0.355
Total alcohol	0 (0, 0.33)	0.6 (0, 0.26)	0.711	0 (0, 0.5)	0 (0, 0.25)	0.898	0.75 (0.25, 3)	1.25 (0.1, 4.5)	0.606
Margarine**	0 (0, 4.5)	0 (0, 7)	0.810	0.13 (0, 4.5)	0 (0, 6)	0.773	0 (0, 4.62)	0 (0, 4.5)	0.653
Butter	1.5 (0, 7)	1.5 (0, 7)	0.781	4.5 (0, 7)	2 (0, 7)	0.943	1.5 (0, 7)	2.25 (0, 7)	0.906
Olive oil based spreading fat	0 (0, 0)	0 (0, 0.44)	0.191	0 (0, 0)	0 (0, 0)	0.582	0 (0, 0)	0 (0, 0)	**0.038**[1]
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	0.5 (0, 1.5)	0.25 (0, 1.5)	0.696
Olive oil	1.5 (0, 3.75)	1.5 (0, 4.5)	0.067	0.13 (0, 3.38)	1.5 (0, 4.5)	**0.038**	1.5 (0, 4.5)	2 (0, 6)	0.244
Animal fat	0 (0, 0)	0 (0, 0)	0.152	0 (0, 0)	0 (0, 0)	0.322	0 (0, 0)	0 (0, 0)	1.000
Salad dressing	0 (0, 0)	0 (0, 0)	0.800	0 (0, 0)	0 (0, 0)	0.538	0 (0, 0)	0 (0, 0)	0.344

Values are median (interquartile range), number of times per week
**p*-values from non-parametric 2 independent samples test (Mann Whitney)
[1]Higher intake in the salmon group (not evident from median values)
**Vegetable oil based spreading fat

Due to the differences observed at baseline for total fish and shellfish consumption between the two groups (median consumption was 0.75 times per week for the control group, and 1.75 times per week for the intervention group, $p = 0.001$) (Table 3.4), the different FFQ fish categories were further explored (Table 3.6). At week 20 the salmon group was consuming significantly more frequently non-oily fish (medians 0.5 and 0.25 times per week, for salmon and control groups respectively, $p = 0.005$), and significantly more frequently oily fish (medians 0.25 and 0 times per week, for salmon and control groups respectively, $p < 0.001$). The proportion of women with a baseline intake of 0.5 times of oily fish per week (i.e. volunteers who just about fulfilled the criterion of 2 portions per month maximum allowed for inclusion into SIPS) was 34% for the salmon group and 8% for the control group. The proportion of women with a baseline oily fish intake of 0-0.25 times per week was 92% for the control group and 65% for the salmon group (χ^2 test $p = 0.001$). At 34 weeks gestation (during intervention), there were no significant differences in white fish intake between the two groups. More importantly, the results of the 34 weeks FFQ, showed that all volunteers assigned in the salmon group reported having oily fish 1-2 times per week, which was interpreted as 1.5 times per week.

Thus, at 34 weeks gestation the salmon group consumed significantly more frequently oily fish than the control group (medians 1.5 and 0 times per week, for salmon and control groups respectively, $p < 0.001$). Finally, at 3 months post-partum, after intervention, the salmon group continued to have higher oily and non-oily fish consumption frequencies than the control group. Interestingly, at 3 months post-partum 17% of the salmon group continued having oily fish more or equal to 1.5 times per week (i.e. ≥ 1-2 times per week) compared to 0% before intervention. Also, at 3 months post-intervention 23% of the salmon group had 0.5 portions of oily fish per week (i.e. twice per month) compared to 34% before intervention (χ^2 test $p = 0.012$). This may suggest that for a small number of volunteers the intervention had produced a sustainable result, and they continued having oily fish twice per week even after the end of the intervention. No differences were seen between salmon and control groups at any time point for fish fingers and fish dishes, and for shellfish. Figure 3.2 describes the distribution of intake of non-oily fish in the FFQ frequency categories, and Figure 3.3 describes the distribution of consumption of oily fish, for both study groups and at all time points.

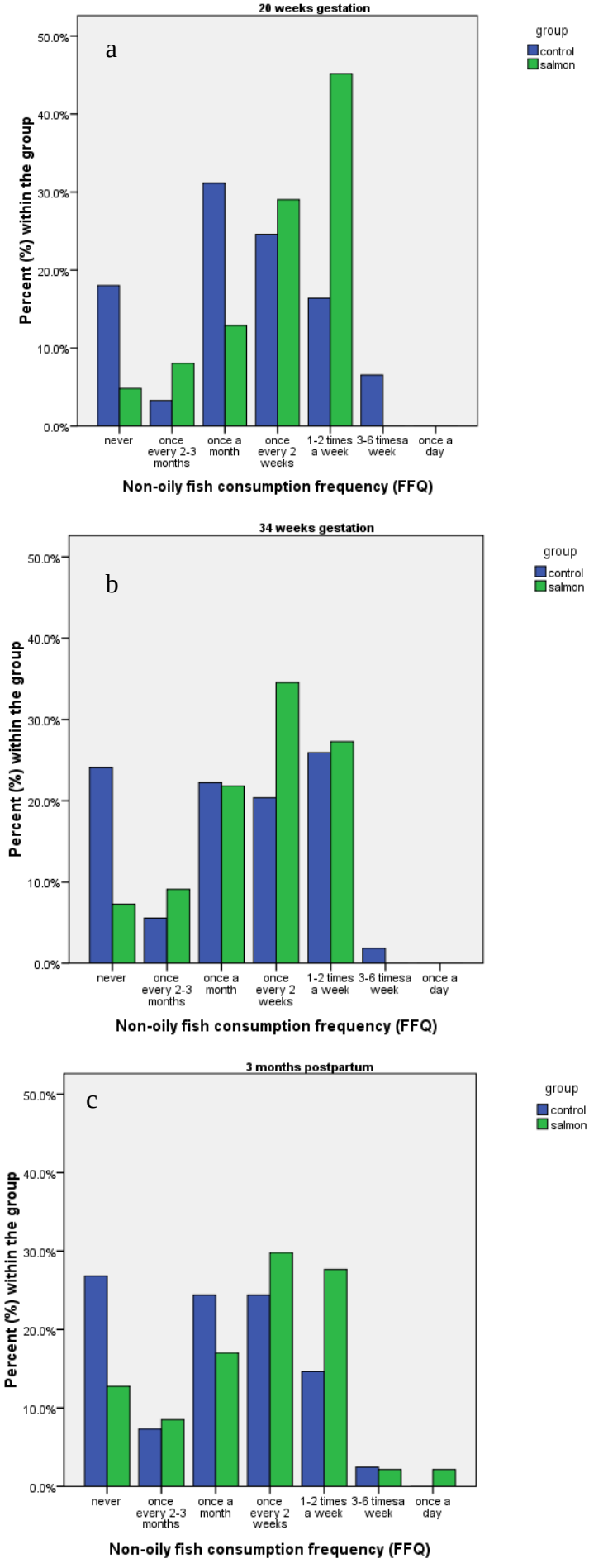
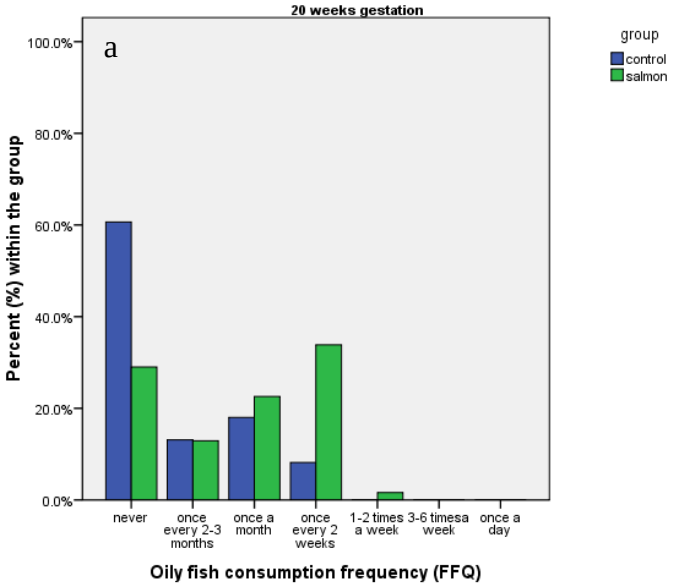
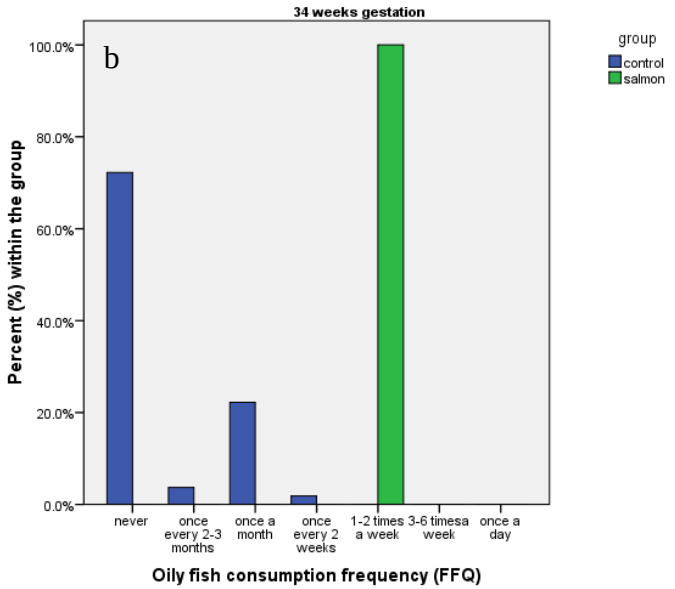


Figure 3.2 Distribution of non-oily fish consumption into the FFQ categories at a) 20 weeks gestation, b) 34 weeks gestation, c) 3 months post-partum





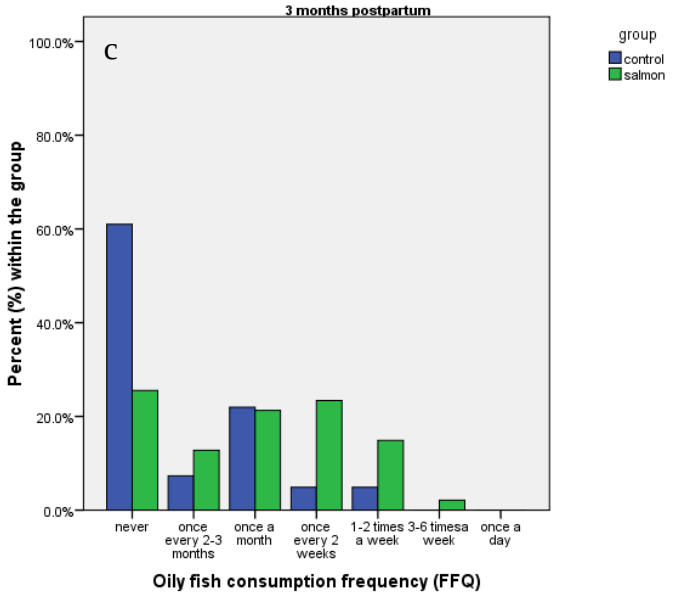


Figure 3.3 Distribution of oily fish consumption into FFQ categories at a) 20 weeks gestation, b) 34 weeks gestation, c) 3 months post-partum

Because of the baseline differences in fish intake between the groups, changes in fish consumption frequencies were calculated: 34 minus 20 weeks gestation, 3 months post-partum minus 20 weeks gestation. The results are shown on Table 3.7. The increase in 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). This change can be seen also on Figure 3.4. The change in oily fish intake was significantly different between the two groups (p<0.001). Also, the increase in total fish consumption in the salmon group (0.85 portions per week more) was significantly different from the almost unchanged total fish consumption frequency from the control group (0.05 times per week less) (p < 0.001). Lastly, there were no statistically significant differences between the groups in fish categories and total fish consumption frequency changes between 3 months post-partum and baseline (20 weeks).

In order to investigate further the red meat between group differences seen at 34 weeks gestation (Table 3.4), all FFQ meat categories were explored. However, no differences in individual meat categories were seen between the two groups (Table 3.6), and the changes of intake of individual meat categories, total red meat and total meat where not different between the two groups (Table 3.7).

Finally, Table 3.8 shows that there were no significant differences in consumption frequencies of functional food products (including products with added LC n-3 PUFA from fish oil) between the groups. For both groups, the majority of the volunteers did not consume these products on a regular basis.

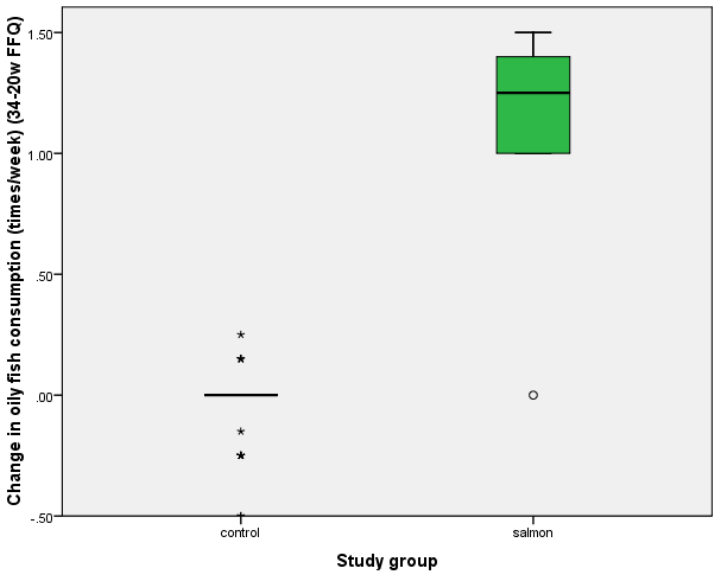


Figure 3.4 Change in weekly oily fish consumption between 34 and 20 weeks gestation in the two study groups
p < 0.001 (Mann Whitney)

Table 3.6 Between group comparisons of weekly consumption frequency of meat and fish categories in the FFQ

Food group	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 54)	Salmon (n = 55)	*p* *	Control (n = 41)	Salmon (n = 47)	*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	0.25 (0, 0.5)	0.5 (0.25, 1.5)	**0.040**
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	0.25 (0, 0.5)	0.1 (0, 0.25)	0.569
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**	0 (0, 0.25)	0.25 (0, 0.5)	<**0.001**
Shellfish	0 (0, 0.1)	0 (0, 0.1)	0.855	0 (0, 0)	0 (0, 0.1)	0.0760	0 (0, 0.17)	0 (0, 0.1)	0.566
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	0.25 (0.1, 0.5)	0.25 (0, 0.5)	0.347
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	1.5 (1, 1.5)	1.5 (0.5, 1.5)	0.619
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	1 (0, 0.5)	0.25 (0, 0.25)	0.812
Beef	0.5 (0.1, 1.5)	0.37 (0.25, 1.5)	0.959	0.5 (0.25, 1.5)	0.5 (0.1, 0.50)	0.351	0.5 (0.17, 1.5)	0.25 (0.1, 1.5)	0.265
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	1.5 (0.5, 1.5)	0.5 (0.25, 1.5)	0.083
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	0.5 (0.25, 1)	0.5 (0.25, 0.5)	0.707
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	1.5 (0, 1.5)	1.5 (0.25, 1.5)	0.806

*p-values from non-parametric 2 independent samples test (Mann Whitney)
Values are median (interquartile range), number of times per week

Table 3.7 Changes in fish and meat weekly consumption frequencies - between group comparisons

Food categories	Change: 34-20 weeks gestation			Change: 3 months postpartum-20 weeks gestation		
	Control (n = 54)	Salmon (n = 55)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
Non-oily fish	0.02 (±0.96)	-0.25 (±0.7)	0.104	-0.22 (±0.92)	0.01 (±1.26)	0.345
Fish fingers and fish dishes	0.01 (±0.38)	-0.11 (±0.43)	0.128	0.04 (±0.41)	-0.12 (±0.57)	0.126
Oily fish	-0.04 (±0.14)	1.21 (±0.26)	<**0.001**	0.05 (±0.32)	0.21 (±0.78)	0.218
Shellfish	-0.04 (±0.27)	-0.25 (±0.7)	0.298	-0.01 (±0.19)	0.07 (±0.24)	0.074
Total fish	-0.05 (±1.07)	0.85 (±0.085)	<**0.001**	-0.14 (±1.25)	0.17 (±1.63)	0.327
Pork	0.08 (±0.91)	0 (±0.12)	0.149	0.07 (±0.51)	0.04 (±0.7)	0.847
Chicken	0.13 (±1.73)	-0.12 (±0.46)	0.094	0.01 (±1.63)	-0.16 (±1.5)	0.612
Lamb	0.08 (±0.69)	-0.32 (±0.96)	0.320	0.01 (±0.33)	-0.04 (±0.45)	0.619
Beef	-0.04 (±1.38)	-0.03 (±0.37)	0.673	0.15 (±0.53)	0.03 (±0.84)	0.409
Total red meat	0.11 (±2.2)	-0.2 (±1.85)	0.424	0.27 (±0.99)	-0.17 (±1.9)	0.195
Total meat	-0.67 (±4.47)	-0.83 (±3.24)	0.831	-0.01 (±3.37)	-0.63 (±3.37)	0.393

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

Table 3.8 Between group comparisons of functional food consumption recorded on the FFQ

Functional foods	20 weeks gestation			34 weeks gestation			3 months post-partum		
Functional food categories	Control n (%)	Salmon n (%)	***p* ***	Control n (%)	Salmon n (%)	***p* ***	Control n (%)	Salmon n (%)	***p* ***
Probiotics									
none	52 (82.2%)	50 (80.6%)	0.478	43 (79.6%)	45 (81.8%)	0.786	35 (85.4%)	34 (72.3%)	0.303
≤3 portions/wk	4 (6.6%)	8 (12.9%)		4 (7.4%)	5 (9.1%)		3 (7.3)	8 (17%)	
≥4/portions/wk	5 (8.2%)	4 (6.5%)		7 (13%)	5 (9.1%)		3 (7.3%)	5 (10.6%)	
Prebiotics									
none	56 (91.8%)	58 (93.5%)	0.223	50 (92.6%)	53 (96.4%)	0.528	41 (100%)	46 (97.9%)	0.348
≤3 portions/wk	4 (6.6%)	1 (1.6%)		3 (5.6%)	2 (3.6%)		0 (0%)	1 (2.1%)	
≥4/portions/wk	1 (1.6%)	3 (4.8%)		1 (1.9%)	0 (0%)		0 (0%)	0 (0%)	
Symbiotics**									
none	55 (90.2%)	53 (85.5%)	0.668	51 (94.4%)	52 (94.5%)	0.223	38 (92.7%)	42 (89.4%)	0.672
≤3 portions/wk	4 (6.6%)	5 (8.1%)		3 (5.6%)	1 (1.8%)		1 (2.4%)	3 (6.4%)	
≥4/portions/wk	2 (3.3%)	4 (6.5%)		0 (0%)	2 (3.6%)		2 (4.9%)	2 (4.3%)	
Products with added fish oil									
none	59 (96.7%)	58 (93.5%)	0.250	51 (94.4%)	51 (92.7%)	0.437	40 (97.6%)	44 (93.6%)	0.376
≤3 portions/wk	2 (3.3%)	2 (3.2%)		1 (1.9%)	0 (0%)		0 (0%)	0 (0%)	
≥4/portions/wk	0 (0%)	2 (3.2%)		2 (3.7%)	4 (7.3%)		1 (2.4%)	3 (6.4%)	
Symbiotics with added fish oil									
none	59 (96.7%)	60 (96.8%)	0.513	54 (100%)	53 (96.4%)	0.157	40 (97.6%)	47 (100%)	0.282
≤3 portions/wk	2 (3.3%)	1 (1.6%)		0 (0%)	2 (3.6%)		0 (0%)	0 (0%)	
≥4/portions/wk	0 (0%)	1 (1.6%)		0 (0%)	0 (0%)		1 (2.4%)	0 (0%)	

**p*-values from Pearson's χ^2 (chi-square) test

**Symbiotics contain both probiotics and prebiotics in one product

3.3.2 Principal component analysis (PCA)

The PCA results described on Table 3.9 show that the salmon group had a significantly higher 'prudent' diet z-score than the control group (p = 0.033), indicating a 'healthier' dietary pattern for the salmon group at baseline. This is in agreement with the food group differences observed between the two groups at 20 weeks gestation (Table 3.4). This 'healthier' pattern was also observed at 34 weeks gestation, when the salmon group had higher 'prudent' diet z-scores that were even more significantly different from the control group (p = 0.007). Finally at 3 months post-partum, the mean score for the salmon group was not statistically different from that for the control. Moreover, the paired t test showed that within the salmon group there was no statistically significant difference between 20 and 34 weeks gestation, however the 'prudent' diet z-score was decreased significantly from 20 weeks gestation to 3 months post-partum (p = 0.016). Paired t tests did not reveal any difference between time-points within the control group (data not shown on tables).

Table 3.9 Prudent diet z-score calculated after applying the SWS correlation coefficients from the principal component analysis (PCA) of the FFQ food categories (Table 3.1))

	20 weeks gestation			**34 weeks gestation**			**3 months post-partum**		
	Control (n = 61)	Salmon (n = 62)	***p* ***	Control (n = 55)	Salmon (n = 54)	***p* ***	Control (n = 41)	Salmon (n = 47)	***p* ***
Applied SWSIPS prudent diet z-score**	-0.193 (±0.768)	0.190 (±1.160)	**0.033**	-0.247 (±0.889)	0.243 (±1.050)	**0.007**	-0.168 (±0.842)	0.147 (±1.107)	0.141

**p*-values from independent samples t-test (normal distribution)
**Values are mean (±standard deviation); Units: sd (± sd)
Z-score= (applied SWSIPS prudent score-prudent score mean)/ prudent score sd
Higher scores indicate a 'healthier diet' as opposed to a 'prudent diet' pattern indicated by lower (negative) scores

3.3.3 Nutrient intakes based upon FFQ

Table 3.10 describes the between group comparisons for total dietary intake of nutrients according to the FFQ at each time point of administration. At 20 weeks of pregnancy the only differences in nutrient intake between the two groups were the statistically significant higher intake of EPA, DHA, and EPA plus DHA in the salmon group (all $p < 0.001$). At 34 weeks gestation intakes of EPA, DHA, and EPA plus DHA were significantly higher in the salmon group compared to the control group (all $p < 0.001$). The same pattern was seen at 3 months post-partum (p = 0.003). However, it must be noted that these are rough estimates for the intake from total diet and supplements as they do not belong to the group

of nutrients declared by SWS to be complete (personal communication with S.M. Robinson). The intake of these LC n-3 PUFA is mainly coming from fish (since none of the subjects consumed fish oil containing supplements) and so it is more appropriate to look at their intake from fish (Table 3.11-Table 3.13) based upon FFQ calculations (see below). Furthermore, although at 20 weeks gestation intake of total n-3 PUFA was not different between the two groups, it was significantly higher in the salmon group at 34 weeks gestation and also at 3 months post-partum. At 34 weeks gestation, the salmon group had a higher daily intake of vitamin B12, selenium, and vitamin D than the control group. At 3 months post-partum, the intakes of these nutrients were not different between the groups. There were no other significant differences between salmon and control groups in intakes of the rest of the macro and micronutrients from total diet at any time point.

According to the FFQ information, there were no differences between the groups in nutritional supplement consumption at baseline and during the intervention. Most importantly, none of the subjects consumed fish oil supplements at baseline and during the intervention, which was in accordance to the inclusion criteria. In chapter 2, it was shown that at baseline (20 weeks FFQ) the majority of the volunteers in both groups (73.8 % control group, 75.8% salmon group; χ^2 (chi-square) p = 0.966) consumed folic acid containing supplements (Table 2.7). During the intervention (34 weeks FFQ), almost equal proportions of subjects either did not consume any supplements (40.7% control group, 47.3% salmon group) or consumed folic acid containing supplements (40.7% control group, 34.5% salmon group), and a lower proportion consumed non-folic acid containing supplements (18.5% control group, 18.2% salmon group) (χ^2 (chi-square) p = 0.762). Post-intervention data (3 month FFQ) showed that there were more women in the salmon group consuming non-folic acid containing supplements (2.4% control group, 19.1% salmon group), whereas the majority in both groups did not consume any supplements (68.3% control group, 48.9% salmon group) (χ^2 (chi-square) p = 0.033).

Table 3.11 - Table 3.13 describe the daily intake of seven nutrients of interest (EPA, DHA, total n-3 PUFA, total n-6 PUFA, selenium, vitamins D and E) from non-oily, oily (including study salmon), and total fish (shellfish and fish dishes), respectively, for both groups at all time points. At 20 weeks gestation, pregnant women in the salmon group had a significantly higher intake of all of these nutrients from non-oily, oily, and total fish. At 34 weeks gestation, there were no statistically significant differences between the two groups in the intake of these seven nutrients from white fish. However, the intakes of all seven nutrients of interest from oily fish (including study salmon) and from total fish were higher for the salmon group compared with control at 34 weeks (all $p < 0.001$). The

median daily intake of LC n-3 PUFA for the salmon group during intervention (34 weeks gestation) was 133.3 mg EPA, 267.2 mg DHA, and 402.6 mg EPA plus DHA, according to FFQ fish consumption frequencies. Finally, at 3 months post-partum, the salmon group had a higher intake of all of these seven nutrients from non-oily, oily, and total fish. The box-plots on Figure 3.5 present the daily intake of EPA, DHA, and EPA plus DHA, from total fish, at all time points and for both groups.

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 (34 weeks gestation minus 20 weeks gestation) (Table 3.14). The mean daily intake of EPA plus DHA from total fish increased by 269.3 mg in the salmon group and for the control group it decreased by 12.2 mg ($p < 0.001$). Moreover, the change in intakes of EPA, DHA, total n-3 PUFA, total n-6 PUFA, selenium, vitamin D and vitamin E from total fish were significantly different between groups (all $p < 0.001$, except for selenium $p = 0.011$). The changes in intakes of LC n-3 PUFA between 20 and 34 weeks gestation are represented on Figure 3.6.

Table 3.10 Between group comparisons of daily intake of selected nutrients from total diet calculated from FFQ at 20 weeks, 34 weeks gestation, and 3 months after delivery (reflecting the 3 months previous to FFQ administration)

Nutrients	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p*	Control (n = 54)	Salmon (n = 55)	*p*	Control (n = 41)	Salmon (n = 47)	*p*
Energy (Kcal) [1]	2230.5 (±670.6)	2168.9 (±569.1)	0.583	2228.7 (±543)	2184.4 (±532.5)	0.668	2005 (±554.96)	2024.93 (±530.47)	0.864
Energy (KJ) [1]	9392.9 (±2821.8)	9132.5 (±2393.2)	0.582	9387.5 (±2288.6)	9196.5 (±2240.4)	0.661	8448.1 (±2340.5)	8526.79 (±2234.7)	0.872
Carbohydrates (g) [1]	296.9 (±93.6)	279.3 (7±8.8)	0.260	296.5 (±81.6)	279.9 (±76.9)	0.276	257.46 (±76.87)	253.79 (±79.96)	0.827
Protein (g)[1]	80.8 (±24.4)	80.7 (±21.3)	0.968	80.3 (±21.7)	80.6 (±19.3)	0.948	74.86 (±20.22)	74.77 (±16.55)	0.981
Total fat (g) [1]	88.1 (±29.2)	88.7 (±25.9)	0.905	88.1 (±23.7)	90.3 (±23.6)	0.635	80 (±25.46)	83.5 (±22.68)	0.508
SFA (g) [1]	34.2 (±12.6)	33.7 (±12.2)	0.810	33.9 (±10)	34.9 (±11.0)	0.604	30.69 (±11.04)	31.78 (±9.96)	0.629
MUFA (g) [1]	31.1 (±10.4)	32.1 (±9.3)	0.573	31.6 (±9.1)	32.7 (±8.0)	0.514	28.9 (±9.54)	30.84 (±8.33)	0.311
PUFA (g) [1]	15.5 (±5.7)	15.5 (±4.7)	0.994	15.6 (±4.9)	15.3 (±4.7)	0.730	13.92 (±4.59)	14 (4.6)	0.877
Total n-3 PUFA (g)[1]	1.8 (±0.6)	1.9 (±0.5)	0.900	1.9 (±0.6)	2.5 (±0.5)	**<0.001**	1.7 (±0.5)	1.9 (±0.6)	**0.036**
Total n-6 PUFA (g)[1]	13.5 (±5.1)	13.4 (±4.3)	0.902	13.6 (±4.4)	12.9 (±4.3)	0.445	12.1 (±4.2)	11.9 (±4)	0.822
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	16.5 (±5.2)	16.2 (±5.4)	0.781
Sodium (g) [1]	3212 (±905.6)	3109.6 (±804.2)	0.508	3056.6 (±767.5)	2989.3 (±867.6)	0.669	2858.37 (±708.48)	2848.4 (±736.62)	0.949
Potassium (g) [1]	3470.1 (±1002.3)	3526.4 (±1050.8)	0.762	3439.1 (±925.7)	3529.8 (±895)	0.604	3257.53 (±905.84)	3224 (±827.4)	0.857
Calcium (mg) [2]	1134.9 (±431.7)	1166.8 (±517.4)	0.711	1199.1 (±429.1)	1200.1 (±403.5)	0.990	1008 (±360)	1037.5 (±358.3)	0.701

Nutrients	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p*	Control (n = 54)	Salmon (n = 55)	*p*	Control (n = 41)	Salmon (n = 47)	*p*
Magnesium (mg) [2]	348.3 (±105.3)	355 (±101.6)	0.722	341.6 (±101.9)	355.2 (±96.2)	0.475	315 (±99.5)	309 (±86.8)	0.741
Phosphorus mg) [2]	1484.9 (±451.7)	1504.2 (±469)	0.816	1513.5 (±428.1)	1533.7 (±390.7)	0.797	1374.6 (±392.5)	1376.6 (±344)	0.980
Zinc (mg) [2]	12.8 (±5.5)	12.8 (±5.6)	0.942	12 (±5.4)	12.3 (±5.4)	0.796	10.8 (±6)	10 (±3.8)	0.435
Retinol (µg) [2]	447.2 (242)	450.5 (234)	0.940	430 (±172)	417.5 (±155.1)	0.693	450 (±211.1)	473.8 (±237.9)	0.625
Retinol equivalents (µg) [1]	361.1 (±200.4)	357.3 (±189.9)	0.915	355.1 (±167.8)	327.1 (±131.9)	0.334	353.7 (±150.2)	347.7 (±176.8)	0.867
Carotene (µg) [2]	3119.7 (±1254)	3230.3 (±1431.9)	0.650	2846.6 (±1195)	3008.1 (±1216)	0.486	3118.5 (±1342.1)	2790.2 (±1366)	0.26
Thiamin (B1) (mg) [2]	2.49 (±1.18)	2.43 (±1.05)	0.789	2.23 (±1.08)	2.29 (±1.05)	0.768	1.97 (±1.05)	1.87 (±0.77)	0.607
Riboflavin (B2) (mg)[2]	2.64 (±1.13)	2.68 (±1.08)	0.861	2.64 (±1.13)	2.68 (±0.96)	0.821	2.23 (±1.04)	2.16 (±0.8)	0.722
Niacin (mg) [2]	25.5 (±10.8)	26.2 (±9)	0.685	24.8 (±10.5)	26 (±10.6)	0.554	23.1 (±9)	21.4 (±7.2)	0.348
Vitamin B6 (mg) [2]	3.96 (±2.92)	4.02 (±2.98)	0.913	3.50 (±2.72)	3.74 (±2.73)	0.649	2.92 (±2.99)	2.64 (±1.64)	0.583
Vitamin B12 (µg) [2]	5.84 (±2.44)	5.94 (±2.46)	0.823	5.91 (±2.65)	7.15 (±2.17)	**0.009**	5.14 (±2.73)	5.36 (±2.2)	0.678
Folate (µg) [2]	564.9 ±590.6)	555.8 (±632.3)	0.935	399.1 (±199)	409.4 (±196.9)	0.784	333 (±168.6)	326.6 (±132.3)	0.843
Vitamin C (mg) [2]	163.6 (±80.4)	173.4 (±82.3)	0.508	176.5 (±159.8)	179.7 (±106.3)	0.902	125.3 (±59.5)	134.2 (±54.4)	0.465
Selenium (µg) [2]	52.4 (±20.4)	56.4 (±16.8)	0.244	49.0 (±17.0)	58.2 (±16.7)	**0.005**	48 (±17.4)	51.1 (±22.6)	0.482
Vitamin E (mg) [3]	15.7 (±7.1)	15.5 (±6.8)	0.934	14.3 (±6.7)	15 (±7.2)	0.592	11.64 (±6.77)	11.21 (±5.16)	0.738
Iron (mg) [3]	15.68 (12.6, 25.76)	15.44 (12.13, 24.28)	0.495	20.25 (11.04, 30.93)	15.46 (11.22, 27.24)	0.641	11.73 (10.36, 16.25)	12.38 (10, 22.69)	0.667
Vitamin D (µg) [3]	4.13 (2.46, 10.11)	3.55 (2.75, 9.96)	0.590	2.9 (2.02, 12.37)	5.75 (5, 9.51)	**<0.001**	2.9 (2, 4.8)	3.18 (2.13, 5.41)	0.369
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**	14.2 (2.4, 41.9)	53.4 (10.6, 89.6)	**0.003**
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**	20.9 (3.8, 74.6)	100.5 (19.4, 158.7)	**0.003**
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**	35.1 (6.2, 116.5)	153.9 (29.9, 248.3)	**0.003**

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

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

[3] Nutrient for which supplements contributed to intake (diet plus supplement intake). Values are medians (interquartile range), *p*-values from non-parametric 2 independent samples test (Mann Whitney) (not normal distribution)

EPA and DHA: the FFQ cannot provide reliable data on intake from the whole diet (incomplete values)
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 3.11 Between group comparisons of daily nutrient intake from non-oily (white) fish consumption recorded on the FFQ

Nutrients	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p**	Control (n = 54)	Salmon (n = 55)	*p**	Control (n = 41)	Salmon (n = 47)	*p**
Total n-3 PUFA (mg)	22.9 (22.9, 45.9)	45.9 (22.9, 137.6)	**0.005**	22.9 (6.9, 137.6)	45.9 (22.9, 137.6)	**0.204**	22.9 (0, 45.9)	45.9 (22.9, 137.6)	**0.040**
Total n-6 PUFA (mg)	36 (36, 72)	72 (36, 216)	**0.005**	36 (10.8, 216)	72 (36, 216)	**0.204**	36 (0, 72)	72 (36, 216)	**0.040**
EPA (mg)	4 (4, 7.9)	7.9 (4, 23.8)	**0.005**	4 (1.2, 23.8)	7.9 (4, 23.8)	**0.204**	4 (0, 7.9)	7.9 (4, 23.8)	**0.040**
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**	8.3 (0, 16.5)	16.5 (8.3, 49.5)	**0.040**
Selenium (µg)	2.79 (2.79, 5.57)	5.57 (2.79, 16.71)	**0.005**	2.79 (0.84, 16.71)	5.57 (2.79, 16.71)	**0.204**	2.79 (0, 5.57)	5.57 (2.79, 16.71)	**0.040**
Vitamin D (µg)	0.071 (0.071, 0.141)	0.141 (0.071, 0.424)	**0.005**	0.071 (0.021, 0.424)	0.141 (0.071, 0.424)	**0.204**	0.071 (0, 0.141)	0.141 (0.071, 0.424)	**0.040**
Vitamin E (mg)	0.044 (0.044, 0.089)	0.089 (0.044, 0.267)	**0.005**	0.044 (0.013, 0.267)	0.089 (0.044, 0.267)	**0.204**	0.044 (0, 0.089)	0.89 (0.044, 0.267)	**0.040**

Values are medians (interquartile range); **p*-values from non-parametric 2 independent samples test (Mann Whitney)

Table 3.12 Between group comparisons of daily nutrient intake from oily fish* consumption recorded on the FFQ

Nutrients	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p**	Control (n = 54)	Salmon (n = 55)	*p**	Control (n = 41)	Salmon (n = 47)	*p**
Total n-3 PUFA (mg)	0 (0, 106.3)	106.3 (0, 212.5)	**<0.001**	0 (0, 58.4)	762.9 (762.9, 762.9)	**<0.001**	0 (0, 106.3)	106.3 (0, 212.5)	**<0.001**
Total n-6 PUFA (mg)	0 (0, 21.3)	21.3 (0, 42.5)	**<0.001**	0 (0, 11.7)	495 (495, 495)	**<0.001**	0 (0, 21.3)	21.3 (0, 42.5)	**<0.001**
EPA (mg)	0 (0, 26.4)	26.4 (0, 52.9)	**<0.001**	0 (0, 14.5)	122.1 (122.1, 122.1)	**<0.001**	0 (0, 26.4)	26.4 (0, 52.9)	**<0.001**
DHA (mg)	0 (0, 48.4)	48.4 (0, 96.8)	**<0.001**	0 (0, 26.6)	248.6 (248.6, 248.6)	**<0.001**	0 (0, 48.4)	48.4 (0, 96.8)	**<0.001**
EPA+DHA from oily fish (mg)	0 (0, 74.8)	74.8 (0, 149.6)	**<0.001**	0 (0, 41.2)	370.7 (370.7, 370.7)	**<0.001**	0 (0, 74.8)	74.8 (0, 149.6)	**<0.001**
Selenium (µg)	0 (0, 1.36)	1.36 (0, 2.71)	**<0.001**	0 (0, 0.75)	9.21 (9.21, 9.21)	**<0.001**	0 (0, 1.360	1.36 (0, 2.71)	**<0.001**
Vitamin D (µg)	0 (0, 0.482)	0.482 (0, 0.964)	**<0.001**	0 (0, 0.265)	3 (3, 3)	**<0.001**	0 (0, 0.482)	0.482 (0, 0.964)	**<0.001**
Vitamin E (mg)	0 (0, 0.053)	0.053 (0, 0.106)	**<0.001**	0 (0, 0.029)	0.879 (0.879, 0.879)	**<0.001**	0 (0, 0.053)	0.053 (0, 106)	**<0.001**

*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); **p*-values from non-parametric 2 independent samples test (Mann Whitney)

Table 3.13 Between group comparisons of daily nutrient intake from total fish* consumption recorded on the FFQ

Nutrients	20 weeks gestation			34 weeks gestation			3 months post-partum		
	Control (n = 61)	Salmon (n = 62)	*p*-value	Control (n = 54)	Salmon (n = 55)	*p*-value	Control (n = 41)	Salmon (n = 47)	*p*-value
Total n-3 PUFA (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**	58.9 (13.5, 182.6)	221.7 (5.11, 379.8)	**0.002**
Total n-6 PUFA (mg)	90.8 (47.4, 252.9)	219.3 (105, 271.6)	**0.001**	91.5 (18.6, 237.7)	600.6 (551, 710)	**<0.001**	75.3 (16, 146.1)	126.8 (56.9, 278.5)	**0.035**
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)	**<0.001**	11.1 (2.4, 39.3)	53.4 (10.6, 89.6)	**0.002**
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)	**<0.001**	20.8 (3.8, 70.1)	100.1 (19.4)	**0.002**
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)	**<0.001**	31.9 (6.2, 110.5)	153.9 (29.9, 245.4)	**0.002**
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)	**<0.001**	4.14 (1.31, 10.59)	7.68 (3.83, 19.88)	**0.021**
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)	**<0.001**	0.141 (0, 0.624)	0.906 (0.141, 1.389)	**0.001**
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)	**<0.001**	0.089 (0, 0.161)	0.17 (0.056, 0.384)	**0.006**

*Total fish including non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish. Values are medians (interquartile range); **p*-values from non-parametric 2 independent samples test (Mann Whitney)

Table 3.14 Changes in daily nutrient intake from total fish* consumption recorded on the FFQ

Nutrients	Change: 34-20 weeks gestation		
	Control (n = 54)	Salmon (n = 55)	***p*****
Total n-3 PUFA (mg)	-16.5 (±120.6)	611.8 (±110.6)	**<0.001**
Total n-6 PUFA (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)	-0.182 (±10.923)	4.534 (±7.861)	**0.011**
Vitamin D (µg)	-0.069 (±0.437)	2.365 (±0.488)	**<0.001**
Vitamin E (mg)	-0.010 (±0.185)	0.772 (±0.123)	**<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; ***p*-values from independent samples t-test

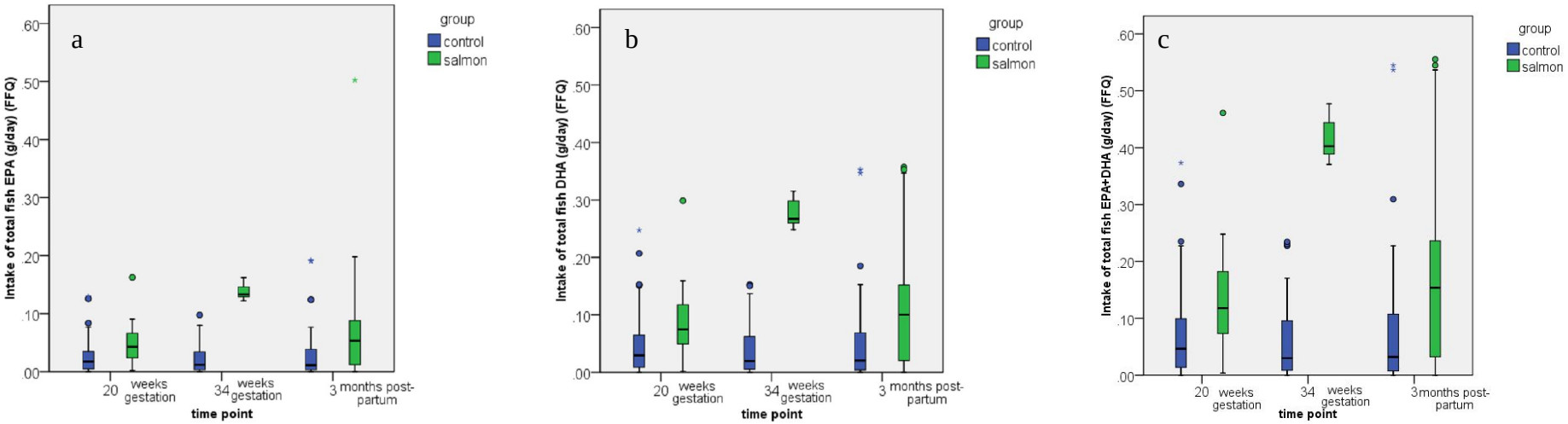


Figure 3.5 Box-plots presenting daily intake of total fish a) EPA, b) DHA and c) EPA plus DHA, at all time points (FFQ)
All $p \leq 0.002$ between groups (Mann Whitney)

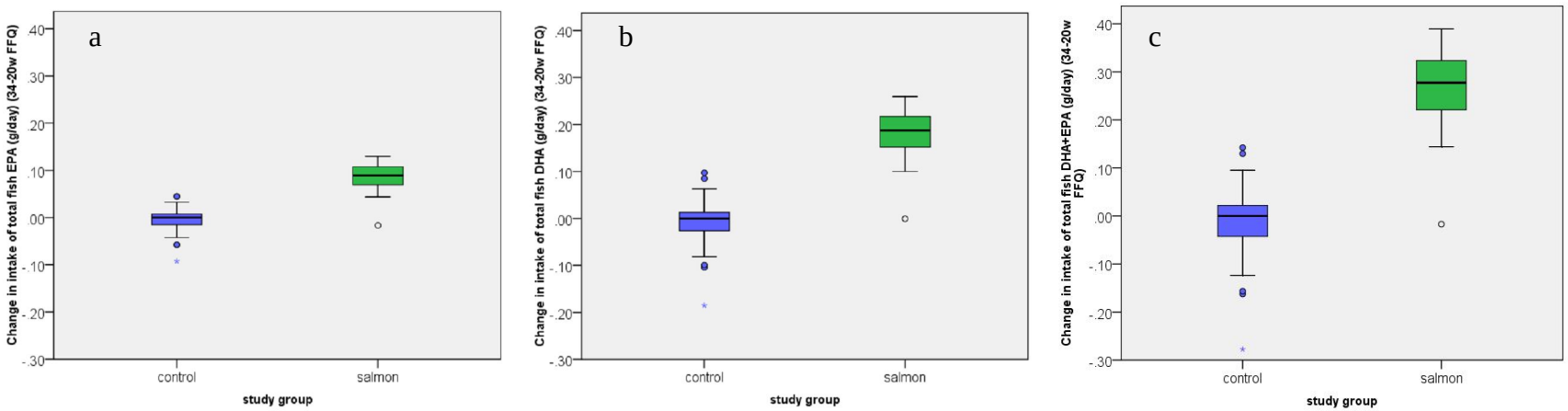


Figure 3.6 Box-plots presenting the change in intake of a) EPA, b) DHA, c) EPA plus DHA, from total fish, between 34 and 20 weeks gestation (FFQ)
All $p < 0.001$ between groups (independent samples t-test)

3.3.4 Information from fish diaries

Table 3.15 describes the mean weekly consumption frequency of study salmon and fish categories consumed by both groups during intervention (21 to 38 weeks gestation), according to the fish diaries. It must be mentioned that the majority, but not all subjects, returned the completed fish diaries, and so this data represents only a subset of the study groups (control group n = 47; salmon group n = 49). The median of the weekly consumption frequency of study salmon in the salmon group was 1.94 portions (Figure 3.7 and Figure 3.8), whereas the median of the weekly oily fish consumption frequency from the control group was 0 portions ($p < 0.001$). The salmon group consumed fewer portions of white fish per week (0.17) compared to control group (0.33) ($p = 0.036$). Also, the salmon group consumed fewer portions of fish fingers and fish dishes. Total fish consumption frequency was 2.11 portions per week for the salmon group and 0.47 portions per week for the control group ($p < 0.001$). Figure 3.9 represents the compliance of the salmon group to consuming two portions of study salmon weekly. The fish diaries showed that, 79.6% of the volunteers in the salmon group were having 2 (or more) portions of study salmon per week for 81.25% of the weeks during intervention (data presented cover the period between 21 to 38 weeks gestation). Also, 24.5% of the volunteers in the salmon group had 2 or more portions of study salmon per week, every single week during 21 to 38 weeks gestation. This data shows that the salmon group complied very well with consuming the salmon as requested (2 portions per week). Figure 3.10 shows the weekly consumption of oily, non-oily, and total fish during every week of pregnancy.

Table 3.16 - Table 3.18 describe the daily intakes of the seven nutrients of interest from non-oily, oily (including study salmon), and total fish, for both groups during intervention according to the fish diaries. The salmon group had a lower daily intake for all seven nutrients of interest coming from white fish compared to control group (all $p = 0.036$). Oily fish and total fish provided significantly more nutrients to the salmon group compared to control (all $p < 0.001$). The median daily intake of the fatty acids of interest from total fish was 162 mg EPA, 325.8 mg DHA, and 490.9 mg EPA plus DHA for the salmon group, and 10 mg EPA, 16.2 mg DHA and 24.4 mg EPA plus DHA for the control group, during intervention (all $p < 0.001$) (data represent the period between 21 to 38 weeks gestation). This data can be seen on Figure 3.11. Figure 3.12 shows the daily intake of EPA plus DHA from total fish, for both groups and during every week of pregnancy.

Table 3.15 Between group comparisons of mean weekly consumption frequencies of fish categories recorded in the fish diaries (data on 21-38 weeks gestation)

Fish Categories	21 to 38 weeks gestation		
	Control (n = 47)	Salmon (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[1]	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)	.507
Total fish[2]	0.47 (0.17, 0.8)	2.11 (2, 2.35)	**<0.001**

[1]Including study salmon

[2]Total fish including non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish.
Values are medians (interquartile range) of number of portions/week; **p*-values from non-parametric 2 independent samples test (Mann Whitney) (not normal distribution).

Table 3.16 Between group comparisons of mean daily nutrient intake from non-oily (white) fish consumption recorded in the fish diaries

Nutrients	21 to 38 weeks gestation		
	Control (n = 47)	Salmon (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)	**0.036**
Total n-3 PUFA (mg)	30.6 (5.1, 51)	15.3 (5.1, 30.6)	**0.036**
Total n-6 PUFA (mg)	48 (8, 80)	24 (8, 48)	**0.036**
Selenium (µg)	3.71 (0.62, 6.19)	1.86 (0.62, 3.7)	**0.036**
Vitamin D (µg)	0.094 (0.016, 0.157)	0.047 (0.016, 0.094)	**0.036**
Vitamin E (mg)	0.056 (0, 0.01)	0.03 (0.01, 0.059)	**0.036**

Values are medians (interquartile range) of nutrient units/day; **p*-values from non-parametric 2 independent samples test (Mann Whitney)

Table 3.17 Between group comparisons of mean daily nutrient intake from oily fish* consumption recorded in the fish diaries

Nutrients	21 to 38 weeks gestation		
	Control (n = 47)	Salmon (n= 49)	***p***
EPA (mg)	0 (0, 7.6)	158.3 (152.7, 164.2)	**<0.001**
DHA (mg)	0 (0, 14)	322.2 (310.6, 333)	**<0.001**
Total n-3 PUFA (mg)	0 (0, 30.7)	988.9 (950.1, 1017.1)	**<0.001**
Total n-6 PUFA (mg)	0 (0, 6.1)	641.7 (608.5, 660)	**<0.001**
Selenium (µg)	0 (0, 0.392)	11.94 (11.49, 12.29)	**<0.001**
Vitamin D (µg)	0 (0, 0.139)	3.89 (3.74, 4)	**<0.001**
Vitamin E (mg)	0 (0, 0.015)	1.14 (1.08, 1.17)	**<0.001**

[*]Oily fish includes study salmon

Values are medians (interquartile range) of nutrient units/day; ***p*-values from non-parametric 2 independent samples test (Mann Whitney)

Table 3.18 Between group comparisons of mean daily nutrient intake from total fish* consumption (fish diaries)

Nutrients	21 to 38 weeks gestation		
	Control (n = 47)	Salmon (n= 49)	*p***
EPA (mg)	10 (4.5, 18.3)	162 (156.4, 167.1)	**<0.001**
DHA (mg)	16.2 (7.6, 37.2)	325.8 (317.9, 339.2)	**<0.001**
Total n-3 PUFA (mg)	43.4 (23.2, 98.8)	996.3 (974.4, 1035.3)	**<0.001**
Total n-6 PUFA (mg)	60.3 (16.4, 106.9)	660 (641.7, 691.4)	**<0.001**
Total fish EPA+DHA (mg)	24.4 (12.7, 56.7)	490.9 (474.6, 506.2)	**<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)	1.158 (1.13, 1.194)	**<0.001**

*Total fish includes non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish. Values are medians (interquartile range) of nutrient units/day; **_p_-values from non-parametric 2 independent samples test (Mann Whitney)

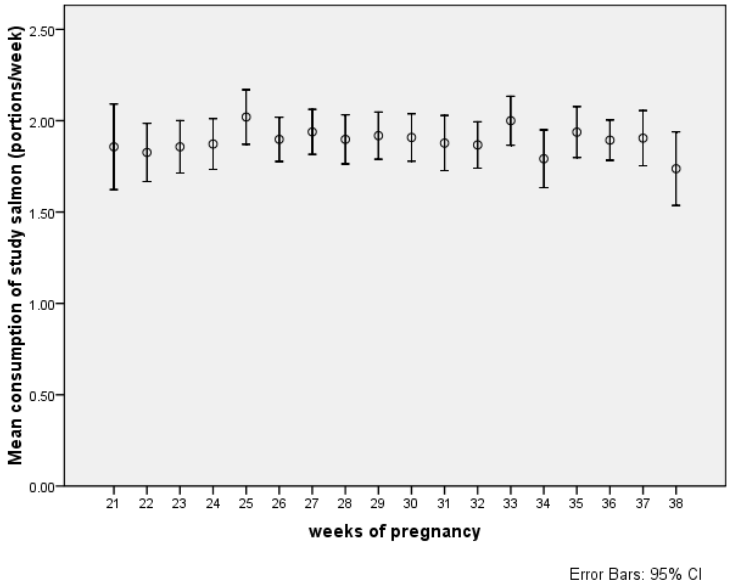


Figure 3.7 Weekly consumption of study salmon in the salmon group for every week of pregnancy

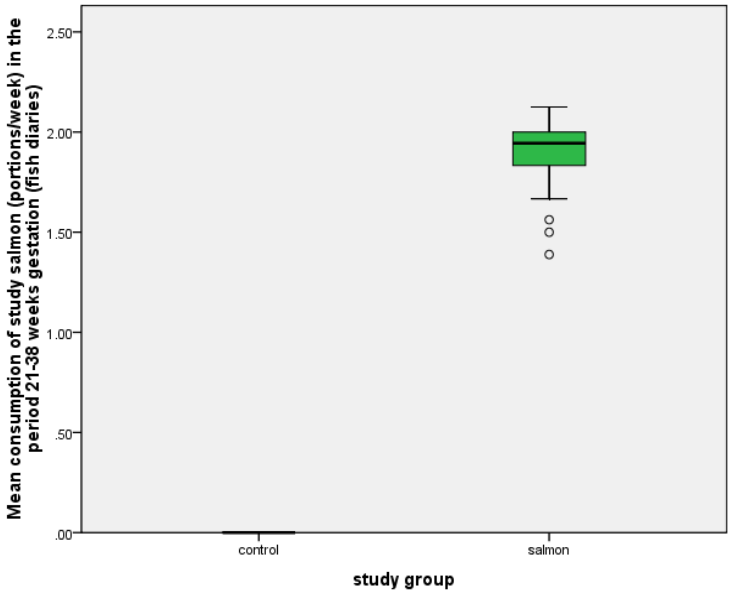


Figure 3.8 Mean weekly consumption of study salmon during the period 21-38 weeks gestation
$p < 0.001$ between groups (Mann Whitney)

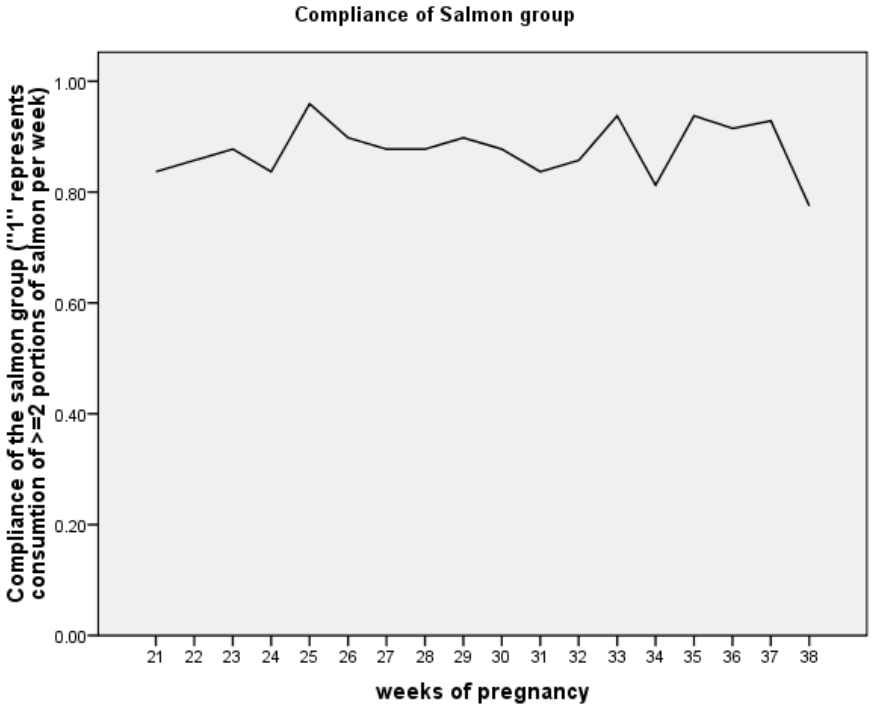


Figure 3.9 Salmon group compliance to consuming ≥ 2 portions of study salmon per week

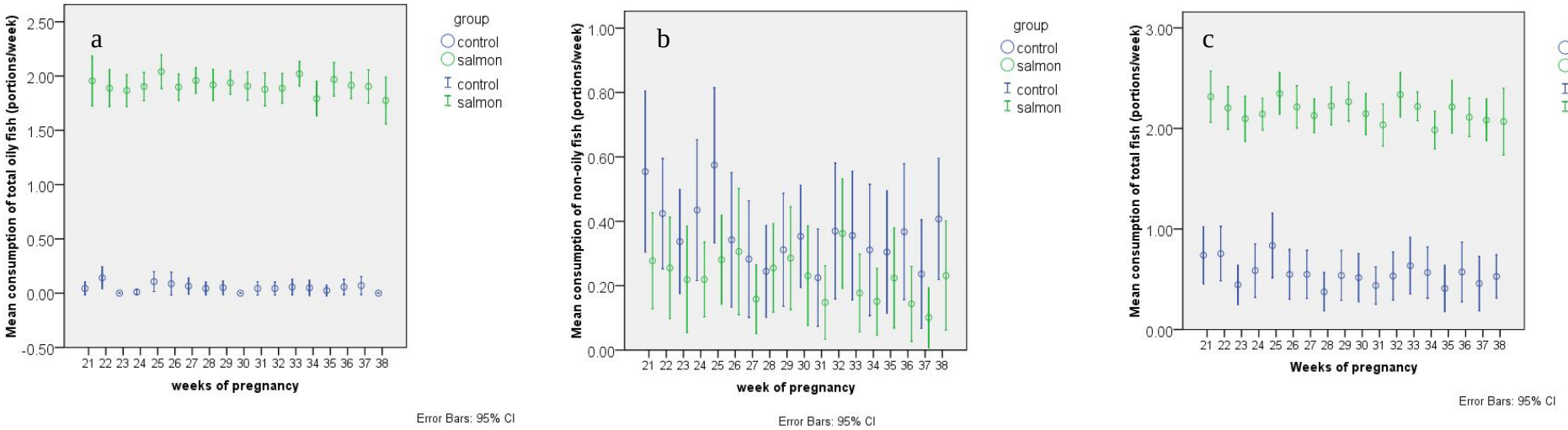


Figure 3.10 Weekly consumption of a) oily, b) non-oily, and c) total fish, for every week of pregnancy

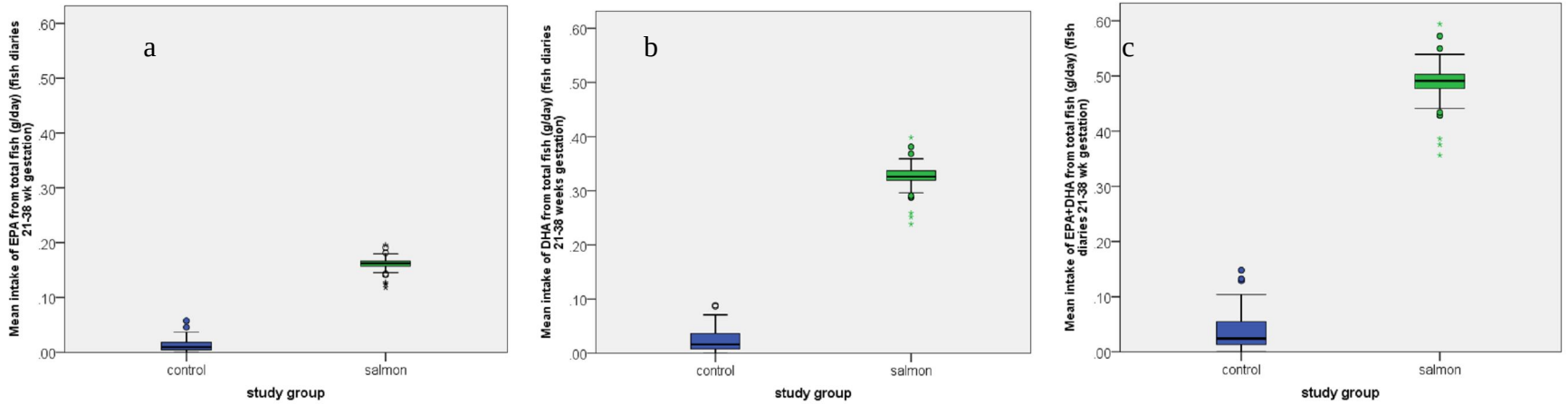


Figure 3.11 Mean daily intake of a) EPA, b) DHA, c) EPA plus DHA, from total fish, during the period 21-38 weeks gestation
All $p < 0.001$ between groups (Mann Whitney)

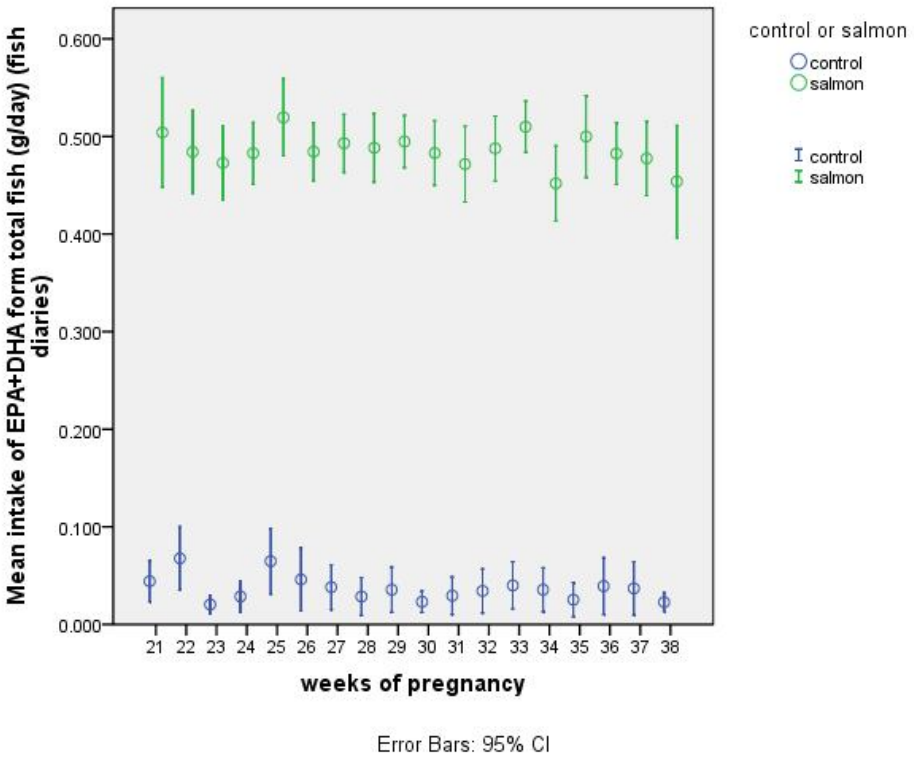


Figure 3.12 Daily intake of EPA plus DHA from total fish, for every week of pregnancy

3.4 Discussion

SIPS is the first dietary intervention with fish in pregnant women. The primary reason for FFQ administration was to determine fish and fish nutrient intake before and during the study, so as to examine the hypotheses that the salmon intervention will result in a) increased intake of oily fish, b) increased intake of specific nutrients (LC n-3 PUFA, vitamin D, Se), c) an improved dietary pattern, and d) a sustained change in diet beyond the end of pregnancy. The data obtained allows each of these hypotheses to be accepted.

According to the FFQ at 34 weeks gestation all subjects (100%) assigned in the salmon group reported having oily fish 1-2 times per week. This result shows that all volunteers in the salmon group may have complied with consuming 2 portions of salmon per week, which falls into the FFQ category 1-2 times per week. Also, at 34 weeks gestation the salmon group consumed statistically significant more frequently oily fish than the control group (medians 1.5 and 0 times per week, for salmon and control respectively, $p < 0.001$). In accordance with this, the fish diary results showed that the median consumption frequency of study salmon from the salmon group was 1.94 portions per week. It has to be pointed out here that a limitation of the FFQ, as with most FFQ, is that it had an ordinal scale of distinct consumption frequency categories, rather than an interval scale providing a 'true' measure (220). The fish diaries were important in determining a more precise consumption frequency. Also, the use of fish diaries as a compliance measure was essential and can be considered as a strength of SIPS. The fish diaries showed that, 79.6% of the volunteers in the salmon group were having 2 or more portions of study salmon per week for the 81.25% of intervention period (data presented cover the period between 21 to 38 weeks gestation).

Also, according to the fish diaries, the salmon group consumed fewer non-oily fish and fish fingers-fish dishes, but had a higher overall total fish consumption during pregnancy. This does not totally agree with the FFQ results which showed that during intervention (34 weeks) the two groups did not differ in non-oily fish and fish fingers/ fish dishes consumption frequencies. The difference in the two methods of assessing fish intake may be again attributed to the limitation of the FFQ to identify small differences because of the distinct consumption frequency categories.

However, at the same time, the use of this specific FFQ in the current study was a strength as it has been validated for use in pregnant women in Southampton (219), it reflected a long term period (presiding 3 months), and it included distinct fish categories.

Although FFQ are designed for larger populations, they have the advantage of being cheap to administer and also of having the ability to summarise data over a long period of time and so to describe habitual diet (229). Epidemiological studies investigating the effect of maternal fish intake during pregnancy on atopic outcomes in the offspring have also used FFQ as a method for determining intake of different types of fish (154-159). These studies are reviewed in section 1.5 and summarised on Table 1.5 (103). Prospective, open-ended dietary methods such as food diaries are demanding to fill in. Food diaries, when completed by motivated volunteers may provide a good source of information. However, in the general population, food diaries are poorly kept (12). In SIPS, the combination of the FFQ with the fish diary is considered as a strength.

Also it is worth mentioning that FFQ may be limited in determining nutrients accurately enough to investigate nutrient-disease associations (230, 231). However, the FFQ used in this study has been validated for calculating nutrients. Crozier *et al.* (214) compared the SWS FFQ dietary pattern data with a 4 day prospective food diary, and concluded that FFQ data provide useful information on dietary patterns in large populations. According to a sub-analysis of the SWS cohort (219), median maternal intake of energy during early pregnancy was 2329 Kcal/day and during late pregnancy 2314 Kcal/day. Median intake of total fat was 91.2 g/day during early pregnancy and 93.2 g/day during late pregnancy. These figures, as well as those for the rest of the nutrients presented by Godfrey *et al.* (219), are very close to the ones calculated here for the SIPS volunteers, showing that the FFQ was a reliable tool to estimate nutrient intake although the study group had a small size. Also, these similarities along with the fact that (as discussed in the methods of this chapter) SIPS correlation coefficients from PCA correlated very well with the SWS coefficients shows that, apart from the SIPS subjects having a low intake of oily fish and being atopic, they are very similar to the average Southampton population and representative of that.

The FFQ data at 34 weeks gestation showed that, during intervention, the intake of EPA, DHA, and EPA plus DHA from oily fish (including study salmon) and from total fish was higher for the salmon group compared to control (all $p < 0.001$). The median daily intake of LC n-3 PUFA from total fish for the salmon group during intervention was 133.3 mg EPA, 267.2 mg DHA, and 402.6 mg EPA plus DHA, whereas for the control group it was 11.9 mg EPA, 19.7 mg DHA, and 30.1 mg EPA plus DHA, according to FFQ fish consumption frequencies at 34 weeks. On the other hand, the results from fish diaries gave slightly higher values of intake for the salmon group and lower for the control. Specifically, the median daily intake of the fatty acids of interest from total fish was 162

mg EPA, 325.8 mg DHA, and 490.9 mg EPA plus DHA for the salmon group, and 10 mg EPA, 16.2 mg DHA and 24.4 mg EPA plus DHA for the control group, during intervention (all $p < 0.001$). This intake covers the recommendation for minimum intake of EPA plus DHA during pregnancy (108% of 450 mg/day). Also, DHA intake in the salmon group covered more than the minimum recommended intake for DHA (200 mg/day) (19).

This difference in intakes calculated by the two different methods of fish intake assessment is associated with the FFQ limitation in calculating nutrients because of its distinct consumption frequency categories. Consumption of salmon twice per week was recorded as consumption 1-2 times per week, and was interpreted as 1.5 times per week for calculations. Also, a limitation of this specific FFQ is that it 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 containing supplements during pregnancy. Also, only a minority of subjects consumed fish oil containing functional foods and this did not differ significantly between the two groups. When calculating 'total diet' EPA and DHA intake (Table 3.10), fish oil contained in functional products was taken into account and no substantial differences with EPA and DHA intake from total fish were observed. Thus, it can be suggested that intake of EPA and DHA from total fish would be a very good estimate of total intake of these LC n-3 PUFA from the whole diet. The use of FFQ to assess micronutrient intake and especially LC n-3 PUFA has been validated in studies on pregnant (232, 233) and non-pregnant subjects (234, 235) and has also been reviewed (236). These studies support that the intake of EPA and DHA can be adequately estimated by FFQ.

An advantage of the fish diaries as opposed to the FFQ, is that they provided exact information of the quantity and types of fish consumed, and therefore consumption frequencies were not distinct categories but a 'true' measure. Also, volunteers filled in the diaries on a daily bases and so the recall bias associated with the FFQ was minimised. However, a disadvantage of the fish diaries is that they have not been validated. Also, fish diaries may have been subject to over or under reporting, as with any other dietary record method, especially when filled in for such a long period of time (12). Finally, the fish diaries were filled in only by a subset of subjects and not by the whole study group.

The use of a FFQ allowed the investigation of other changes that may have occurred in the dietary pattern of the subjects associated with the intervention. However, a too long FFQ may be a limitation especially when conducting larger studies. Crozier *et al.* (237)

showed that a 20-item FFQ based on the foods that characterise the SWS 'prudent' diet pattern can be used as an alternative tool to summarise the diets of young women in Southampton. Also, the 20-item 'prudent' diet score was correlated with red blood cell folate similarly to the 100-item SWS FFQ score. The use of a shorter FFQ may be helpful when there is interest in only one aspect of the diet, such as vitamin D and calcium or fat (237), or fish intake and LC n-3 PUFA. Generally, a shorter FFQ, especially designed to determine consumption of food rich in the nutrients that are of interest can be less time consuming and equally efficient.

There have been no studies of fish intervention during pregnancy. However the current study may be compared to other studies which investigated the effect of increased fish consumption on different health outcomes and in different populations (238-241). Unlike the current study, these studies used food diaries as a method for measuring compliance and dietary intake. Specifically, the study of Pot *et al.* (241, 242) investigated the effects of increased fish consumption on colorectal cancer markers. The intervention period was 6 months, and volunteers were randomly allocated to receive dietary advice plus either two portions per week of oily fish (salmon) (n=82), or non-oily fish (cod) (n=78), or just dietary advice (n=82). According to the article, salmon and cod provided 1.4 and 0.09 g of LC n-3 PUFA per day, respectively. Volunteer compliance was checked by food diaries and by regular phone calls every 2-4 weeks. Before the start of the intervention fish consumption was on average 1.5 times per week. Fish consumption increased by 1.4 times per week for the salmon group and 1.3 times per week for the cod group. The study of Din *et al.* (238) examined the effect of increased oily fish on platelet-monocyte aggregation. Fourteen volunteers were asked to consume 500 g of mackerel per week for 4 weeks (providing the equivalent of approximately 1 g of EPA plus DHA per day). The control arm did not receive a dietary intervention. Subjects were asked to keep a 3-day weighed food diary at baseline, during intervention, and after intervention. The study of Vidgren *et al.* (243) included dietary fish and fish oil intervention in a university setting. Subjects were asked to consume 5 portions of fish per week and diet was assessed with a 4-day record at baseline and three 4-day records during intervention. Also, the records of the University restaurant were used to identify types of fish consumed. In the dietary fish group intake of EPA was 380 mg/day and of DHA 670 mg/day.

Borland *et al.* (215) reported a median intake of total fish and shellfish of 1.5 times per week and 1.8 times per week for two measures made two years apart in young women in Southampton. The more recently published SWS data (218) reported a median intake of total fish and shellfish of 1.8 and 1.9 times per week for early and late pregnancy

respectively. These figures represent median intakes in the general Southampton female population which is very close to the consumption of total fish and shellfish of the salmon group at baseline (before intervention 1.75 times/week). However, the control group had a much lower level of consumption (0.75 times/week). Subjects were recruited in SIPS on the basis of low self reported oily fish intake.

The baseline difference in intakes of fish and nutrients from fish between the two SIPS groups, despite the fact that subjects were randomly assigned to the two arms, is a limitation of the current study. The proportion of women with a baseline intake of 0.5 times of oily fish per week (i.e. volunteers who just about fulfilled the criterion of 2 portions per month maximum) was 34% for the salmon group and 8% for the control group. However, the increase in 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 (decreased by 0.04 times per week) ($p < 0.001$). Also, the mean daily intake of EPA plus DHA from total fish was increased by 269.3 mg for the salmon group and for the control group it remained almost unchanged (slight decreased by 12.2 mg) between before and during intervention ($p < 0.001$).

A strength of the current study is that it explored the intake of different types of fats and oils. The reason why this was explored was because of the particular interest of the study in the effect of dietary intake of LC n-3 PUFA on maternal fatty acid status and immunity. It was essential to make sure that the two groups were not different in terms of intake of different sources of fat, and thus other fatty acids apart from the ones coming from the study salmon. Moreover, it was important to investigate the intake of functional foods with added LC n-3 PUFA, as well as the intake of prebiotic and probiotic products which have been associated with effects on the immune system (244). There were no differences in intake of these products between the groups.

An issue of interest, which was not primarily targeted by the intervention (at least initially), was its impact on the intake of nutrients other than the LC n-3 PUFA. According to the nutrient content of salmon, such nutrients of interest would be vitamin D, vitamin E, selenium, total n-3 PUFA, total n-6 PUFA. It was shown from both the fish diaries and the FFQ, that the salmon group had a higher intake of vitamin D, selenium, and total n-3 PUFA from oily fish and total fish at 34 weeks gestation compared to the control group. More importantly, this increased intake form fish was reflected in an increased intake of these nutrients from total diet (estimated by FFQ). Total diet selenium intake was 58.2 µg/day for the salmon group (97% of Reference Nutrient Intake (RNI; 60 µg/day)) and 49

μg/day for the control group (82% RNI) ($p = 0.005$) (18). The contribution of study salmon to total diet intake of selenium was 20% which is quite substantial. The intake of the control group was very close to the average intake that has been recently reported by the National Diet and Nutrition Survey (NDNS) in the UK (39 μg/day) (13). It has to be noted that total diet selenium intake should be treated as a rough estimate, since the content of selenium in foods depends on soil content and data are not completely reliable (222). Total diet vitamin D intake was 5.75 μg/day for the salmon group (57% RNI; RNI for pregnant women is 10 μg/day) and 2.9 μg/day for the control group (29% RNI) ($p < 0.001$) (18). It is worth noting that the study salmon contributed to 68% of the total vitamin D intake in the salmon group. Total diet vitamin D intake in the control group was comparable to that reported by NDNS (2.7 μg/day) (13). As mentioned in the introduction, early fish and vitamin D exposure improves bone health in infancy and childhood (207, 208). Maternal vitamin D intake during pregnancy has been associated with decreased risk of sensitization to food allergens in the offspring (209) and evidence shows the importance of vitamin D and selenium in reducing risk of atopic disease development (92, 210-213). Also, according to the 34 week FFQ data, total diet vitamin B12 intake was also higher in the salmon group compared to the control group ($p = 0.009$). Din *et al.* who conducted an intervention with mackerel, showed with dietary records that the mackerel group had higher intakes of iodine, selenium, vitamin B12, and vitamin D during intervention (238). The study of Vidgren *et al.* (243) showed that consumption of 5 fish meals per week increased vitamin D intake (9 μg/day) according to three 4-day dietary records (243). These results are in agreement with the results of SIPS reported here, although nutrient intake was measured by FFQ. The study of Pot *et al.* (241, 242), mentioned earlier, involved intervention in non-pregnant women with oily fish (salmon), or non-oily fish (cod), or just dietary advice. It was shown that serum selenium and vitamin D were not significantly increased in the fish consumption groups compared to control (241). This may be due to the content of selenium and vitamin D in the fish provided by that study, or also by the intake of these nutrients from other sources and sun exposure. In SIPS, it would be expected that the blood levels of selenium and vitamin D would be higher in the salmon group compared to the control group based on the fact that intake was significantly and substantially higher. Finally, vitamin E and total n-6 PUFA intake from oily and total fish at 34 weeks gestation were significantly higher for the salmon group compared to the control group (reflected by FFQ and fish diaries). However, the contribution of fish to the intake of these nutrients from total diet was small and it did not result in a significant increase in the intake of these nutrients (vitamin E and total n-6 PUFA) from total diet (FFQ data).

An interesting question to be addressed, through the dietary information available in SIPS, was whether the salmon intervention resulted in changes of dietary patterns during the intervention. During the intervention, according to the findings of the 34 weeks gestation FFQ, the salmon group had a statistically significant lower intake of red meat compared to the control group. Also, within the salmon group, chicken intake was significantly reduced from 20 to 34 weeks gestation, and so was the intake of non-oily fish. Also, fish diaries showed lower intake of non-oily fish and fish dishes in the salmon group compared to the control group during intervention. Total meat consumption was decreased significantly from 20 to 34 weeks gestation within the salmon group. As a result of the above, it can be speculated that increased salmon consumption was compensated by a decreased consumption of other fish (non-oily fish and fish dishes according to fish diaries) and also by a decrease in red meat, chicken, and total meat intake. In contrast to our findings, the fish intervention of Pot *et al.* (241, 242) found that dietary habits other than fish consumption did not change in any of the intervention groups and specifically that meat consumption remained constant. It is difficult to explain how fish could be incorporated into the diet of the subjects in the study of Pot *et al*., without a reduction in intake of some other component such as red meat or chicken. This may be related to the use of food diaries that do not reflect long periods of time and such a change in dietary patterns cannot be observed.

The PCA results described showed that the salmon group had a statistically significant higher 'prudent' diet z-score than the control group, indicating a 'healthier' dietary pattern for the salmon group, at baseline and at 34 weeks gestation. The higher 'prudent' diet scores in the salmon group, reflecting 'healthier' dietary patterns, are in accordance with the higher fish consumption both at baseline and at 34 weeks. The higher scores might be a true reflection of the group's diet, however it could be attributed to misreporting from the volunteers of the salmon group as their answers might have been affected by the fact that they were 'chosen' to take part in the study, and allocated to the salmon group. On the other hand, it may be considered that volunteers in the salmon group made an effort to eat more healthily as they were aware of the fact that they were taking part in a nutrition intervention, and they belonged to the salmon group. Many volunteers reported verbally during appointments that they were trying to combine salmon with 'healthier' dietary choices, such as cooking it with vegetables, and generally taking care of their diet in total, as they realised that this would be good for themselves and the baby. At 3 months post-partum, the mean 'prudent' diet z-score did not differ significantly between the two groups. The lower 'prudent' diet scores for the salmon group after intervention may be

associated with them not taking such care of their diet at the same level as they did during pregnancy, or it may be as a result of more realistic reporting compared to before and during intervention.

The information from the 3 month post-partum FFQ was used to investigate whether the intervention had an impact on fish consumption habits after its end. The salmon group had a higher oily and non-oily fish consumption after intervention than the control group. Interestingly, at 3 months post-partum 17% of the salmon group continued having oily fish more or equal to 1.5 times per week (i.e. ≥ 1-2 times per week) compared to 0% before intervention. However, there were no statistically significant differences between the groups in fish categories and total fish consumption frequency changes between 3 months post-partum and baseline (20 weeks). This may suggest that, although the study salmon provision during pregnancy increased oily fish intake during intervention (because of the salmon consumed), there was a sustained effect beyond the intervention period for only for a small number of volunteers. However, for the majority of the volunteers this increase was not sustained after the end of the intervention, as volunteers returned to their habitually low intake of oily fish (65% at 20 weeks gestation and 60% at 3 months post-partum had oily fish 0-0.25 times per week). Similarly, the fish intervention study of Din *et al.* showed that the nutrient intake differences observed during the study (higher iodine, selenium, vitamin B12, vitamin D) due to the intervention were not sustained after the end of the intervention (238). Borland *et al.* (215) examined the stability of dietary patterns in young women over a 2-year period. The study was performed on a subgroup of the SWS and it was concluded that dietary patterns were reasonably stable. This may explain the difficulty in changing dietary patterns and why high oily fish intake was not sustained after the end of SIPS intervention for the majority of the subjects in the salmon group.

A typical nutrition supplement containing fish oil for pregnant women contains 60 mg EPA, 300 mg DHA, 30 µg selenium, and 10 µg vitamin D in a daily dose. According to the findings of the fish diaries, consumption of two portions of study salmon per week (oily fish data including study salmon) provided 158.3 mg EPA, 322.2 mg DHA, 11.94 µg selenium, and 3.89 µg vitamin D per day. This would mean that consumption of two portions of oily fish per week would cover approximately 40% of the amount of vitamin D and selenium, 260% of the amount of EPA, 106% of the DHA, and 133% of total EPA plus DHA provided from a pregnancy supplement. This is the first study in pregnant women to provide this information, supporting the importance of oily fish during pregnancy as they can be an excellent source of a combination of nutrients. This, in relation to the fact that increased oily fish consumption resulted in lower meat intake, can

provide strong evidence in order to support public health messages and interventions that target increased consumption of oily fish (at least two portions) instead of the use of fish oil supplements during pregnancy. Of course all considerations about limiting contaminant intake during pregnancy should be taken into account when choosing the source of fish.

3.5 Summary and conclusions

In conclusion, SIPS successfully increased oily fish intake in the salmon group to the target of 2 portions of study salmon per week. This was reflected by FFQ (all volunteers reported intake 1-2 times per week during intervention at 34 weeks gestation) and fish diaries (median intake of study salmon was 1.94 portions per week). Both dietary assessment methods reported a median oily fish intake of the control group of 0 portions per week. According to FFQ, median daily intake of EPA plus DHA was 403 mg for the salmon group and 30 mg for the control group ($p < 0.001$). The fish diary results showed similar levels of intake (490 mg for the salmon group and 24 mg for the control group) ($p < 0.001$). Moreover, it was shown that increased salmon consumption increased total diet intake of selenium, vitamin D, and total n-3 PUFA. Consumption of two portions of oily fish per week would provide more than the minimum recommended intake of DHA and of EPA plus DHA, 60% of the recommended intake of vitamin D, 20% of the recommended intake of selenium. Furthermore, consumption of two portions of oily fish per week would cover approximately 40% of the amount of vitamin D and selenium, 260% of the amount of EPA, 106% of the DHA, and 133% of total EPA plus DHA provided by a typical pregnancy supplement. Increased salmon consumption was compensated by a decreased consumption of other fish (non-oily fish and fish dishes) and also by a decrease in red meat, chicken, and total meat intake. These results further support the public health messages and interventions that aim to increase oily fish consumption to at least two portions of oily fish per week during pregnancy. Finally, the SIPS intervention resulted in a sustained increased oily fish consumption after the end of the intervention only for a small number of volunteers in the salmon group. The majority of volunteers returned to their habitually low intake of oily fish. Factors associated with long-term sustainable increase of oily fish consumption by women of reproductive age but also for the general population remain to be explored.

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

4.1 Introduction

Apart from being important as energy sources to the body, fatty acids are associated with structural and metabolic functions which primarily involve polyunsaturated fatty acids (PUFA) (245). The human body does not have the ability to synthesise the simplest PUFA with double bonds three (n-3 PUFA) or six (n-6 PUFA) carbon atoms from the methyl terminus of the hydrocarbon chain (see section 1.1). These fatty acids - linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) - must be obtained from the diet and thus they are termed essential fatty acids. Their metabolic derivatives arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) are functionally very important. These fatty acids may be termed long chain (LC) n-3 and n-6 PUFA, and they are conditionally essential. Fatty acids are required by the developing foetus in order to maintain fluidity and structure of membranes, as well as to act as precursors of eicosanoids (prostaglandins, leukotrienes, thromboxanes) (245). LC PUFA (especially DHA and ARA) are important for foetal growth and development (246, 246-248) and they influence length of gestation (249). Perhaps most importantly, DHA is accumulated in high concentration in the membranes of cells of the nervous and visual system (i.e. in foetal brain and in foetal retina) during pregnancy, and this accumulation is very important for visual and cognitive development both before and after birth (250). High amounts of DHA are incorporated into membranes in the brain and retina especially during the last trimester of pregnancy when foetal nervous system growth is very rapid (248). Total foetal DHA accretion *in utero* is approximately 10 g and it takes place mainly in the last 10 weeks of pregnancy, and it has been estimated that foetal accrual of DHA occurs at a rate of about 200 mg/day in late pregnancy, and such accrual continues after birth (Figure 4.1) (245). Most of the DHA is stored in foetal adipose tissue *in utero* (50 times more than in foetal brain) in order to be released after birth and utilised for growth (245). Obviously fatty acids including ARA and DHA are provided to the foetus from the mother via the placenta. There is a specific mechanism of uptake of these LC PUFA across the placenta (245). It is known that preterm birth, which curtails maternal supply of DHA to the foetus, is associated with sub-optimal neural and visual development (251), which can be improved by providing exogenous DHA (252-254). LC n-3 PUFA have also been shown to influence immune function (7, 38, 94) and it is now considered that supply of EPA and DHA to the foetus might be important in promoting appropriate immune development (47, 87, 88, 93). However this has been little studied in intervention studies, and those studies that have been conducted have been in the context of influencing immune development in a way that would reduce risk of allergy in the infant (22, 178, 182, 183,

186, 188). Thus, studies have demonstrated that maternal fish intake or fish oil supplementation during pregnancy has a protective effect against allergy, asthma and atopy in the offspring (see section 1.5, (103)).

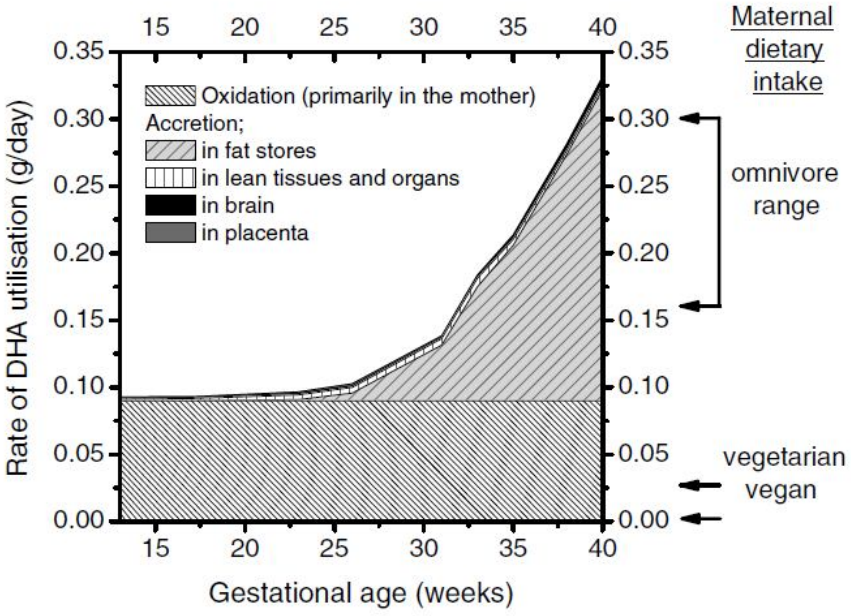


Figure 4.1 Docosahexaenoic acid (DHA) oxidation in the mother and accretion in the foetal brain, adipose tissue, lean tissues, and placenta
Figure taken from Haggarty (245). The DHA content of the foetal lean tissue and organs was assumed to be the same as the placenta. The rate of accretion in the brain and placenta is very small relative to other components. The right y axis shows the reported daily dietary intake of DHA in different groups.

Fatty acids of chain length of about 8 carbons or more, including LC n-3 and n-6 PUFA, are insoluble in plasma as they are highly hydrophobic. In order to allow their transport in the bloodstream they are esterified to form more complex lipid forms including triglycerides (TAG), phospholipids, and cholesteryl esters (CE) and these are assembled along with proteins into large, soluble structures termed lipoproteins. In TAG, three fatty acids are bound, each by an ester bond, to the glycerol backbone. One fatty acid bound by an ester bond to the fourth ring of cholesterol forms CE. In phospholipids two fatty acids are bound, each by an ester bond, to the sn-1 and sn-2 positions of the glycerol backbone; the sn-3 position is bound to a polar head group. Thus most non-cellular fatty acid in the bloodstream is within lipoproteins in the form of TAG, phospholipids and CE; the principal phospholipid in human plasma is phosphatidylcholine (PC). TAG, PC and CE represent transport pools of fatty acids typically carrying fatty acids from the gut or liver to peripheral tissues and back. PC is also a major component of cell membranes along with other phospholipids. The fatty acid composition of plasma PC is similar to that of cell membrane PC. Thus the fatty acid composition of plasma PC can also be taken to reflect that of a functional fatty acid pool, i.e. cell membrane phospholipids, although this is of

course only an approximation. Fatty acids are also present in plasma in non-esterified form (termed non-esterified fatty acids (NEFA)) bound non-covalently to albumin. The composition of fasting plasma NEFA reflects adipose tissue fatty acids (245, 255) released during hydrolysis of TAG in adipose tissue.

During pregnancy there are two principal metabolic stages. During the first two trimesters foetal growth is limited and the mother accumulates nutrients and energy in her body stores. The last trimester is characterised by a catabolic phase whereby maternal adipose tissue lipolysis releases fatty acids to the maternal circulation which will be transferred to the foetus through the placenta (256). Maternal NEFA released from lipolysis are mainly re-esterified into TAG in the liver, and these are released into the circulation in the form of very low density lipoprotein (VLDL). There is an increase in plasma TAG concentration during late gestation and this is termed hyperlipidemia of pregnancy (256). Also, during late pregnancy levels of TAG increase in low density lipoproteins (LDL) and high density lipoproteins (HDL) probably associated with increased activity of the cholesteryl ester transfer protein (CETP) (257). This enzyme controls TAG and CE exchange between VLDL, LDL, and HDL. Maternal hypertriglyceridemia is considered to be beneficial for the foetus, as it may enhance the availability of important fatty acids from the mother. During late pregnancy, intestinal absorption of TAG is very efficient and TAG are carried in the form of chylomicrons in maternal plasma (255).

The function of the placenta and many physiological and metabolic adaptations taking place during pregnancy play a fundamental role in optimising the transport of fatty acids to the foetus (245). Complex mechanisms are involved in placental fatty acid transport (247). Lipoproteins can be taken-up by the placenta by lipoprotein receptors existing on the placenta. Also, because of the expression of lipoprotein lipases (LPL) on placenta, maternal TAG can by hydrolysed and the NEFA released can by taken up by the placenta, which can be re-esterified and stored and then hydrolysed and released into the foetal bloodstream (256). Transport of LC PUFA to the placenta is mainly done by uptake of maternal TAG intact in plasma lipoproteins (see Figure 4.2 taken from Herrera (258)). This is greater than uptake of NEFA from placenta.

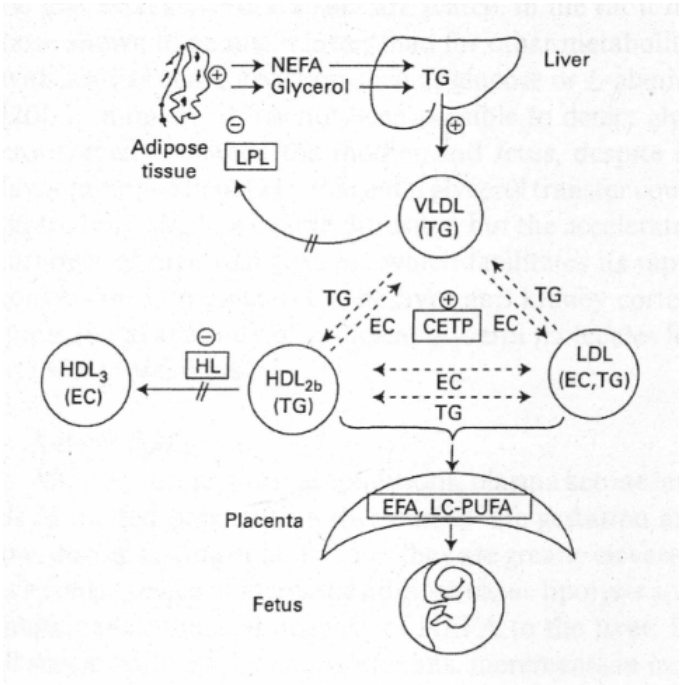


Figure 4.2 Lipid metabolism during pregnancy and placental lipid transfer
Figure taken from Herrera (258). LPL, lipoprotein lipase(s); HDL, high density lipoprotein(s); VLDL, very low density lipoprotein(s); LDL, low density lipoprotein(s); TG, triglyceride(s); EC, esterified cholesterol; EFA, essential fatty acid(s); LC-PUFA, long-chain polyunsaturated fatty acid(s); CETP, cholesteryl ester transfer protein(s)

However, maternal plasma NEFA are also a source of LC PUFA for the foetus (257). Thus, fatty acids to be transported by the placenta are derived mainly from TAG in chylomicrons or VLDL, from which they are released by TAG hydrolase/LPL before entering the placenta. The placenta uptake of circulating TAG is concentration gradient dependent. Also, as mentioned above, maternal NEFA bound to albumin or lipoproteins bound to apoprotein receptors are sources of fatty acids for the placenta (248). The demands for cholesterol by the foetus are relatively high, especially during the last trimester of gestation. In the early stages of gestation, maternal cholesterol substantially contributes to foetal cholesterol, whereas later on the foetus seems to have the ability to synthesise cholesterol especially in liver and brain (256). The presence of lipoprotein receptors in placenta, enables the placenta to take up fatty acids in the form of CE from maternal lipoproteins, but the maternal contribution to the foetal plasma cholesterol pool seems to be very small (259). Fatty acids present in phospholipids (e.g. PC) in lipoproteins may also be hydrolysed, taken up and resynthesised as TAG or PC in placenta (248).

The presence of membrane fatty acid binding protein in human placenta results in preferential transfer of certain fatty acids to the foetus. There is preferential transfer of LC PUFA, mainly DHA, as opposed to the essential fatty acid precursors (247, 248). Because the placenta lacks Δ6- and Δ5-desaturase activity for the conversion of essential fatty acids (linoleic and α-linolenic) to LC PUFA and foetal enzyme activity *in utero* is very low, the

foetus depends on placental LC PUFA transfer (245, 248). Accretion of LC PUFA *in utero* results in higher concentration of DHA and ARA in the foetal adipose tissue compared to maternal (Figure 4.3) (245).

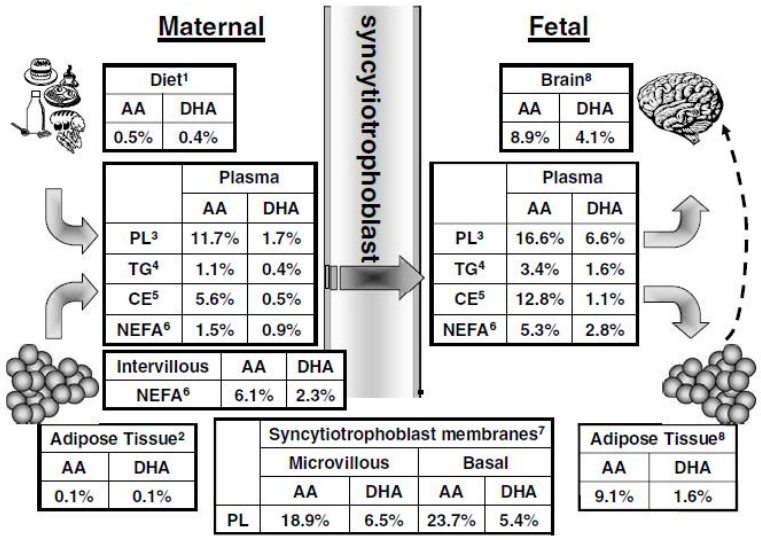


Figure 4.3 Arachidonic acid (AA) and docosahexaenoic acid (DHA) as a proportion of total fatty acids in the mother and foetus

Figure taken from Haggarty (245). Data are shown for the diet of pregnant mothers, the adipose tissue, maternal and cord blood plasma phospholipids (PL), triglyceride (TG), cholesteryl ester (CE) and NEFA, the placental microvillous and basal membranes, and adipose tissue and brain at birth.

De Vriese *et al.* (260) showed that maternal and cord plasma at delivery had a different CE and PC fatty acid profile, with umbilical cord plasma exhibiting a higher concentration of ARA and DHA, indicating a preferential supply to the foetus with the LC PUFA needed for development. In the study of Berghaus *et al.* (261) higher proportions of DHA and ARA were found in cord plasma in almost all lipid fractions compared to maternal plasma, indicating the selective mobilisation through placenta and accretion of these from the mother to the foetus. Burdge *et al.* (262) showed enrichment of 18:3n-3 in plasma TAG and of DHA in plasma PC in pregnant women, which reflects hepatic regulation of n-3 PUFA metabolism during pregnancy and may act to enhance the delivery of DHA to the placenta. Min *et al.* (263) showed that pregnancy is associated with a decline in percentage levels of ARA and DHA in maternal plasma TAG, which may indicate the selective transfer of these fatty acids to the foetus.

Houwelingen *et al.* (264) found that maternal plasma PC fatty acids expressed as a percentage (i.e. each fatty acid as a percentage of total fatty acids) were highly significantly correlated with foetal plasma PC fatty acids. Vlaardingerbroek & Hornstra (265) concluded that maternal fatty acid percentages at delivery in red blood cell and

plasma PC were highly correlated with neonatal fatty acid percentages at birth. Also, De Vriese *et al.* (266) showed that maternal plasma PC EPA and DHA percentages were positively associated with intake of these fatty acids during pregnancy. Also, EPA and total n-6 PUFA levels in cord plasma PC were positively correlated with maternal dietary intake of these fatty acids during pregnancy. Similarly, another study showed that maternal intake of EPA plus DHA during pregnancy (determined by FFQ) correlated significantly with maternal and cord LC n-3 PUFA blood levels. Also, maternal LC n-3 PUFA levels were associated with cord blood levels (267). Placenta fatty acid content might reflect the ability to supply fatty acids to the foetus, and its analysis may be a good indicator of placenta fatty acid transfer (247, 248). The placenta lacks the enzymes Δ5- and Δ6-desaturase needed for conversion of essential fatty acids to ARA, EPA and DHA. Also, the foetus has limited capacity to synthesise these fatty acids because of limited desaturase activity (245, 248). This results in the foetus being dependent on maternal supply of these fatty acids (247, 248), and thus, maternal dietary intake of LC PUFA is related to the availability of these fatty acids to the foetus (245). Studies in healthy non-pregnant volunteers have shown that plasma concentrations of EPA and DHA can be useful biomarkers for the assessment of habitual dietary intake of these fatty acids (268, 269).

The results of the most recent NDNS (2008/2009) showed that women aged 19-64 years (including non-consumers) consume on average 7 g/day (49 g/week) coated and fried white fish, 15 g/day (105 g/week) other white fish, shellfish and tinned tuna, and 11 g/day (77 g/week) of oily fish. The same report, showed that amongst female consumers of oily fish (29% of women) aged 19-64 years daily consumption was 39 g/day (273 g/week or 2 portions/week) (13). The most recent NDNS data (13) did not report on intake of LC n-3 PUFA. However, the previous NDNS results (12) showed that mean intake of EPA plus DHA for adults in the UK was 244 mg/day, from fish and animal sources (12). However, since about 70% of the adult population do not eat oily fish, this is an overestimate of true average intakes which are likely to be 100 mg/day (14) or less (15). Also, according to NDNS (2008/2009) 2% of the population aged 19-64 years are vegetarian (13). However, it has been previously shown that the percentage of women in the UK who are vegetarians is higher than this (around 11-12%) (12). Exclusion of meat and fish can result in low intake of EPA and DHA among pregnant vegetarian women. The intake of DHA in vegetarian pregnant women has been estimated around 10-30 mg/day (16, 17).

There are no published intervention trials with fish during pregnancy. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish (in this case farmed salmon) in pregnant women. SIPS specifically focuses on pregnant women whose

offspring are at high risk of developing atopic disease, with one aim being to identify whether there is an effect on atopy outcomes in the offspring. As described in Chapter 2, a total of 123 pregnant women were randomly assigned to either consume 2 portions of farmed salmon per week from week 20 of pregnancy until delivery or to continue their habitual diet low in oily fish (less or equal to 2 portions per month). The aim of the work described in this chapter is to investigate the effect of the intervention on the fatty acid status of the women enrolled in SIPS during pregnancy and on that of their placenta and umbilical cord tissue at delivery. It is considered that the fatty acid composition of maternal plasma and of placenta will determine the provision of fatty acids to the foetus affecting its fatty acid status and consequently its developing immune system in a protective way though the provision of LC n-3 PUFA (and other key nutrients) from the study salmon.

The primary hypotheses examined by the work described in this chapter are that:

- increasing habitually low intake of oily fish (≤ 2 portions per month) to 2 portions per week during pregnancy, will increase maternal plasma lipid levels of EPA and DHA, and that these levels will be higher than in the control group;
- the salmon intervention will increase maternal peripheral blood mononuclear cell (PBMC) levels of EPA and DHA, and that these levels will be higher than in the control group;
- the salmon intervention will result in higher placenta and umbilical cord tissue EPA and DHA levels in the salmon group compared to the control group.

4.2 Study design, materials and methods

4.2.1 Study design, subjects, subject characteristics and the intervention

In chapter 2 the study design, the recruiting procedure, the nature of the women recruited and their characteristics, and the nature of the intervention were all described in detail. One group of pregnant women (n = 61) did not undergo any dietary intervention and maintained their habitual diet which was low in oily fish. A second group of pregnant women (n = 62) consumed two portions of farmed salmon per week from week 20 of pregnancy until delivery, which corresponds to the recommended oily fish intake for pregnant women in the UK (1-2 portions per week) (11). Full details of the salmon are given in section 2.4: salmon provided 1.73 g EPA plus DHA per portion (i.e. 3.46 g per week or 494 mg/day), which would result in intake higher than the UK minimum recommendation of 450 mg/day (11). Also, it has been recommended that DHA intake should be at least 200 mg/day in women of reproductive age (19). Subjects were seen in the fasting state at 20, 32-34 and 38 weeks gestation and blood was drawn to enable fatty acid analysis of plasma and PBMC and measurement of immunological markers (next chapter). At delivery, placenta and umbilical cord tissue samples were collected and analysed for fatty acid status.

4.2.2 Maternal blood collection

Plastic 6 mL lithium heparin (LH) vacutainer blood bottles were obtained from NHS stores, Southampton General Hospital. Maternal blood collection was performed by an adequately trained nurse or member of the SIPS team. At 20, 32-34, and 38 weeks gestation, blood was collected in three 6 mL LH bottles and stored at room temperature for further processing within 8 hours of collection.

4.2.3 Preparation of peripheral blood plasma and mononuclear cells

4.2.3.1 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), and crystal violet were purchased from Sigma-Aldrich. RPMI culture medium (without L-glutamine and antibiotics) was purchased from PAA Laboratories. Acetic acid was purchased from Fisher Scientific. Phosphate buffered saline (PBS) tablets were purchased from Oxoid.

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.

4.2.3.2 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 later fatty acid analysis and to be used for culturing PBMC (see chapter 4).

PBMC 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 PBMC number, 2×10^6 cells were removed and frozen at -80˚C for fatty acid analysis. The remaining PBMC were prepared for culturing (chapter 4).

4.2.4 Preparation and storage of placenta and umbilical cord tissues

4.2.4.1 Materials

Phosphate-buffered saline (PBS) tablets were purchased from Oxoid.

4.2.4.2 Procedures

Instructions about how to collect the placenta and umbilical cord were given to midwives in the Princess Anne Hospital and other local birth centres, in the form of verbal information and laminated cards. The whole placenta and umbilical cord were collected by the midwife after delivery and stored at 4˚C.

Within 48 hours of collection, a cross-sectional placental sample of full depth, including both amniotic membrane and decidua (approximate size 1 cm × 1 cm × full depth of placenta) was taken from a position where the placenta was the thickest and close to umbilical cord. A 2-3 mm transverse section of the initial placental sample obtained, including amniotic membrane and decidua, was washed in PBS, blotted dry and stored at -80˚C for fatty acid analysis.

A 2-3 mm transverse section of umbilical cord from a position close to the placenta was taken, wrapped in aluminium foil and stored in liquid nitrogen for fatty acid analysis.

4.2.5 Fatty acid analysis of plasma, peripheral blood mononuclear cells, and placenta and cord tissue by gas chromatography

4.2.5.1 Materials

Sodium chloride (NaCl), butylated hydroxytoluene (BHT), potassium bicarbonate ($KHCO_3$), potassium carbonate (K_2CO_3), heneicosanoic acid, cholesteryl heptadecanoate, tripentadecanoin, and menhaden oil standard fatty acid methyl ester mix were all purchased from Sigma. Dipentadecanoyl PC was purchased from Avanti Polar Lipids. Chloroform, methanol, glacial acetic acid, hexane, ethyl acetate, toluene, sulphuric acid (H_2SO_4) were purchased from Fisher Scientific. Aminopropylsilica solid phase extraction (SPE) cartridges (NH_2) were purchased from Varian. Institute of Human Nutrition (IHN) oil standard mix was an 'in house' preparation using Sigma and Nuchek fatty acid standards (see Appendix M for details of the FAME included in the IHN standard mix).

4.2.5.2 Procedure

Quantitative analysis of fatty acids was performed for plasma samples, and thus a known volume of plasma was used for analysis (0.5 mL). Moreover, internal standards of known concentration and volume (Table 4.1) were added to plasma samples prior to lipid extraction. The plasma with added internal standards was reconstituted with 0.9% NaCl to a final volume 0.8 mL.

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

Lipid class	Shorthand notations of standard fatty acid	Systematic name of standard fatty acid	Concentration of standard solution in hexane	Volume of standard solution added	Amount of standard added
Phosphatidylcholine (PC)	15:0	Dipentadecanoyl PC	1 mg/mL	100 µL	100 µg
Non-esterified fatty acids (NEFA)	21:0	Heneicosanoic acid	0.5 mg/mL	25 µL	12.5 µg
Cholesteryl esters (CE)	17:0	Cholesteryl heptadecanoate	3 mg/mL	50µL	150 µL
Triglycerides (TAG)	15:0	Tripentadecanoin	1 mg/mL	50µL	50 µg

The whole amount of PBMC stored for fatty acid analysis (approximately 2×10^6 cells) was reconstituted with 0.9% NaCl to a final volume of 0.8 mL. Placenta and cord tissue sections (obtained as described above) were mixed with 0.8 mL of ice cold 0.9% NaCl and homogenised using an Ultraturrax. Internal standards were not added to PBMC or tissues, as these were not analysed quantitatively.

Total lipid was extracted from samples with chloroform:methanol (2:1; vol/vol) containing butylated hydroxytoluene as antioxidant. After adding NaCl (1M), the sample was centrifuged and the lower non-aqueous phase was collected. The sample was dried under gas nitrogen at 40°C.

Separation of lipid classes was performed for plasma samples by solid phase extraction (SPE). An aminopropylsilica SPE cartridge was placed on the SPE tank connected to a vacuum pump. The total lipid extract was dissolved in chloroform before applying the sample 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 TAG and CE and were dried under nitrogen gas at 40°C. 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. NEFA were eluted by chloroform:methanol:glacial acetic acid (100:2:2; vol/vol/vol) under vacuum until dry. The fractions were dried under nitrogen gas at 40°C. 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 was applied to the new column. The sample was allowed to drip through under gravity and the remaining liquid was collected under vacuum. The column was then washed twice with hexane under vacuum. The washes containing CE were dried under nitrogen gas at 40°C. TAG 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 purified lipid classes of plasma or the total lipid extract of PBMC or tissues were diluted with toluene. Fatty acid methyl esters (FAME) were formed by adding methanol containing 2% (v/v) H_2SO_4 to the purified lipid classes (for plasma samples) or to the total lipid extract (for PBMC and tissues). The sample was incubated at 50°C for 2 hours. Neutralising solution (0.25M $KHCO_3$, 0.5M K_2CO_3) and hexane were added to the sample. The sample was centrifuged and the upper phase containing the FAME was collected and then dried under nitrogen gas at 40°C. The sample was diluted with hexane and was transferred to a gas chromatography (GC) vial.

The fatty acid composition of each sample was determined by GC in a Hewlett-Packard 6890 gas chromatograph fitted with an autosampler. A 30 m BPX-70 SGE capillary column was used. Helium was used as the carrier gas. The exact running conditions and details of the GC program are described in Appendix N. The detector was a flame ionisation detector (FID). A series of standards run previously (Institute of Human

Nutrition mix (IHN), Menhaden oil) (Figure 4.4 and Figure 4.5) was used to identify individual peaks of FAME. Figure 4.6-Figure 4.12 show the chromatogram for each plasma lipid fraction (PC, TAG, CE, NEFA), PBMC, and placenta and cord tissues. The software used to analyse the chromatograms was Agilent ChemStation.

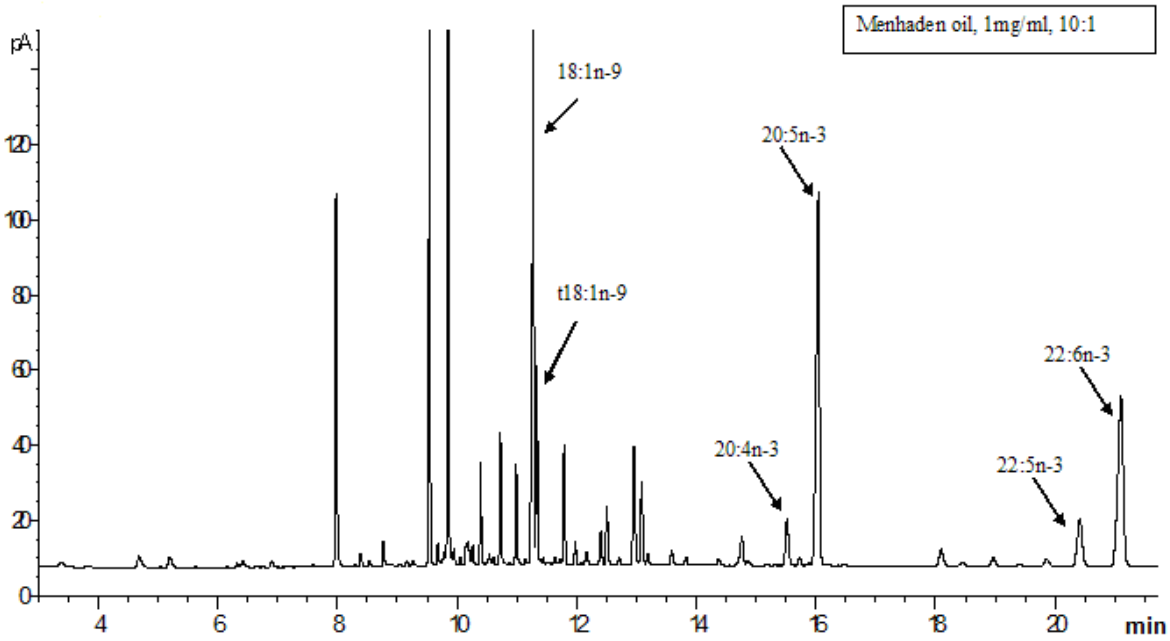


Figure 4.4 Typical GC profile of the Menhaden oil standard

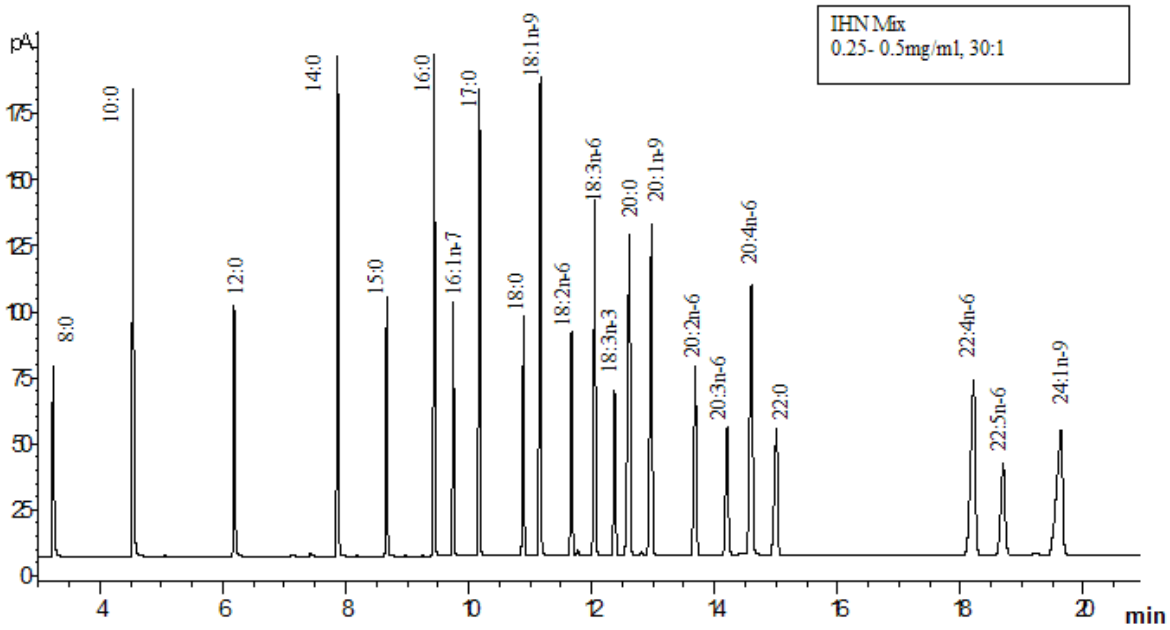


Figure 4.5 Typical GC profile of the Institute of Human Nutrition (IHN) standard mix

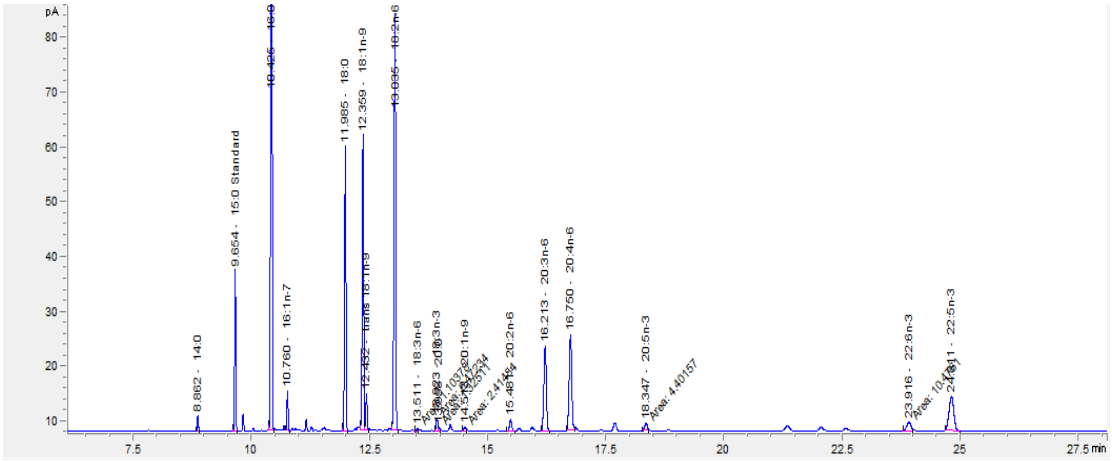


Figure 4.6 Typical GC profile for plasma PC

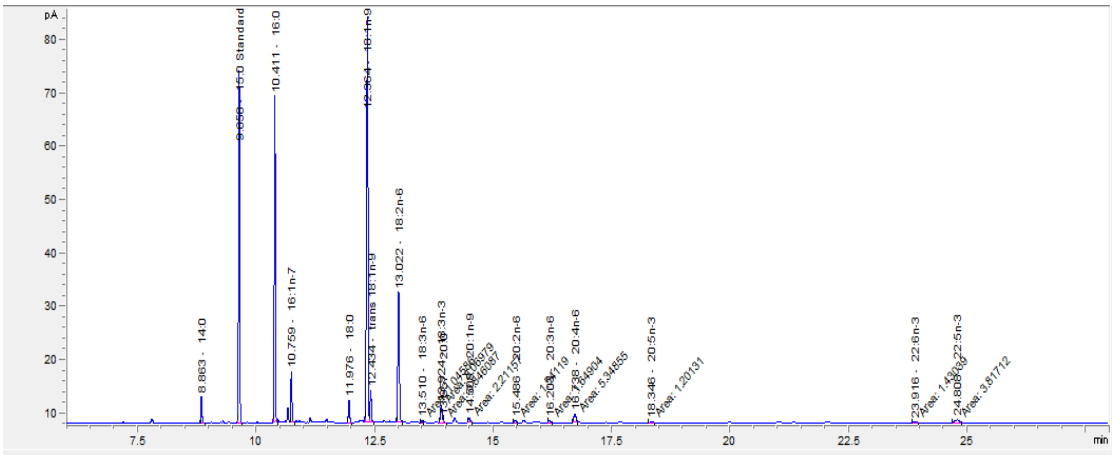


Figure 4.7 Typical GC profile for plasma TAG

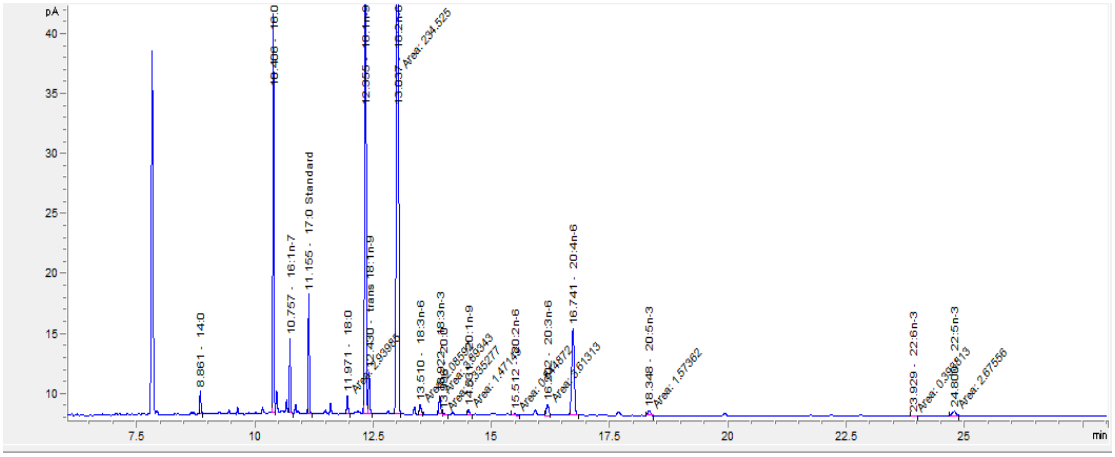


Figure 4.8 Typical GC profile for plasma CE

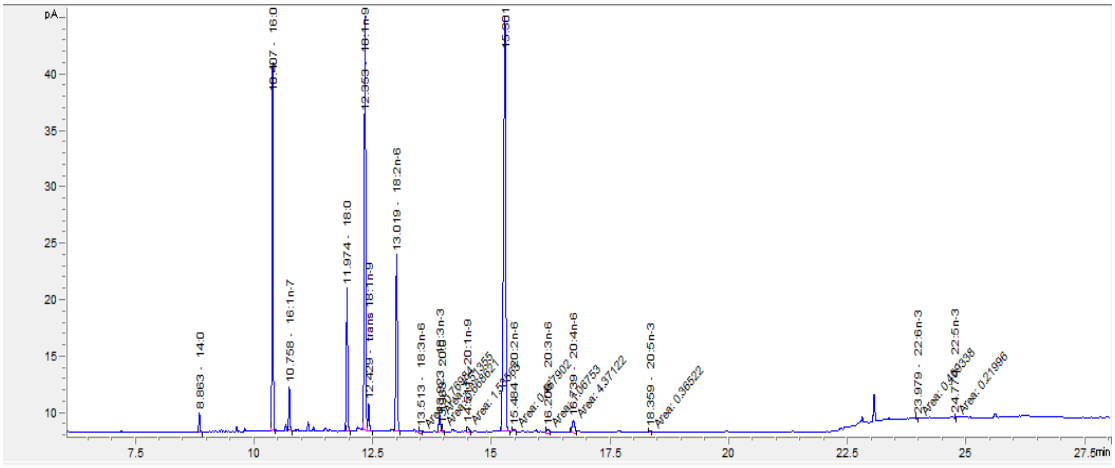


Figure 4.9 Typical GC profile for plasma NEFA

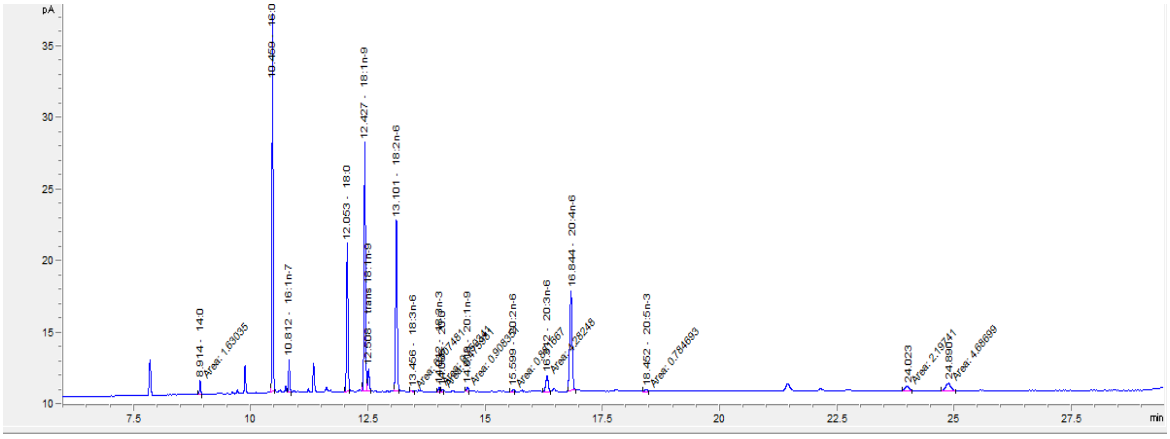


Figure 4.10 Typical GC profile for PBMC total lipids

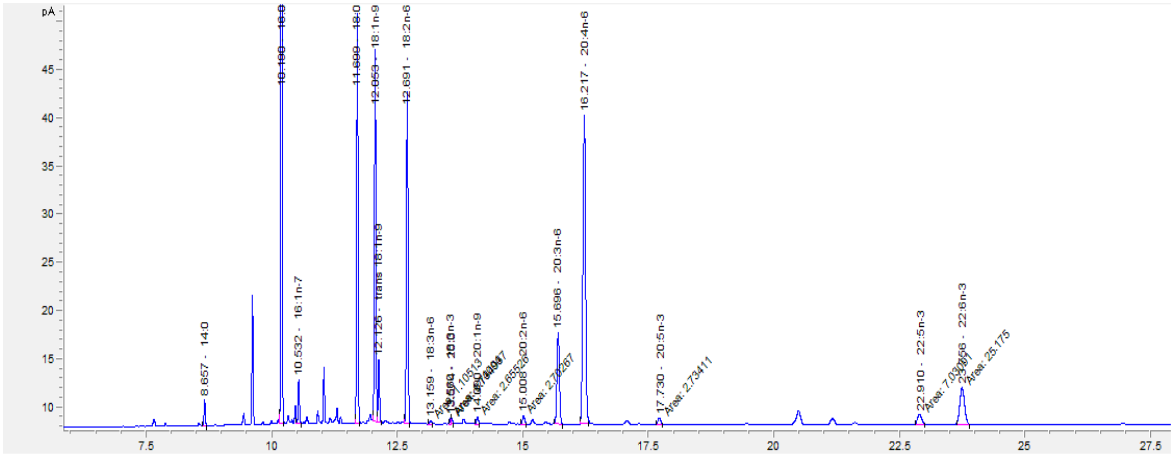


Figure 4.11 Typical GC profile for placenta tissue total lipids

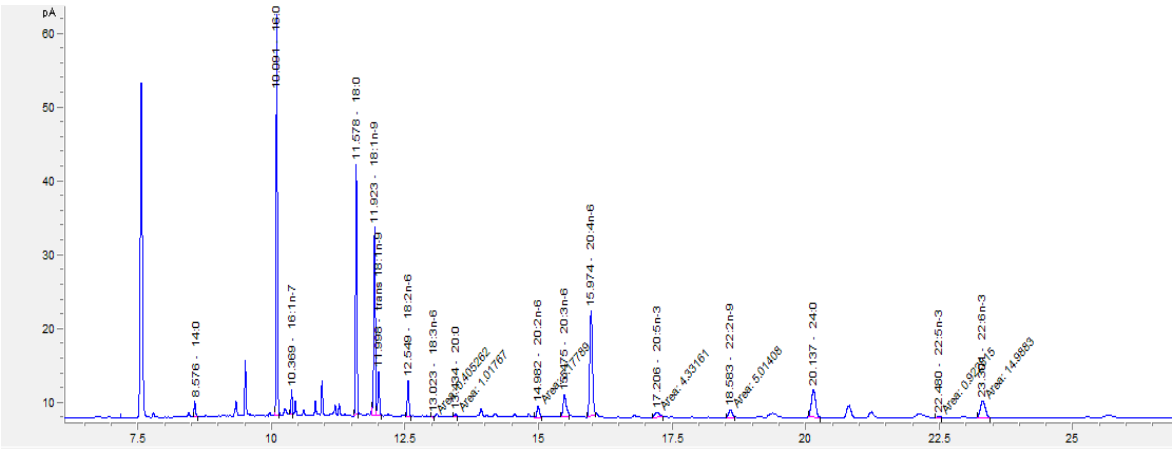


Figure 4.12 Typical GC profile for cord tissue total lipids

4.2.5.3 Fatty acid identification and quantification principles

Qualitative analysis: Each sample run on the GC results in a chromatogram where fatty acids (actually FAME) appear as individual peaks as shown in Figure 4.6 to Figure 4.12. 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 of the GC. The time that each peak of fatty acid appears on the chromatogram is equal to the time that each fatty acid is retained inside the column of the GC until it gets carried by helium to the detector, it is called 'retention time' and it is characteristic for each fatty acid. Typically, FAME with shorter chains and fewer or no double bonds have a shorter retention time than fatty acids with longer hydrocarbon chains and more double bonds, and thus they appear first on a chromatogram. This is attributed to the physical properties of the fatty acids according to their structure. The identification of the specific fatty acid is done by comparison of the retention times of FAME within a known standard mix of fatty acids (IHN and Menhaden oil) with those of FAME in the sample. After the identification of the fatty acid, the area under each peak is calculated and the proportion of each peak area to the sum of all peak areas identified is calculated. This proportion represents the percentage of the fatty acid in the total fatty acids in the sample analysed: total lipids (for PBMC and tissues) or each lipid class (PC, TAG, CE, NEFA for plasma).

Quantitative analysis: Internal standards added to the sample at the beginning of the lipid extraction (see Table 4.1 for plasma internal standards) allow for determination of 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

information along with the known initial amount of sample used in analysis allows for calculations of the absolute concentration of each fatty acid in the sample.

4.2.6 Statistical analysis

All data from fatty acid analysis presented here were normally distributed and thus parametric statistical tests were used. The independent samples t-test was used for between group comparisons at each of the three time points of measurement (20, 34, 38 weeks gestation) and for placenta and cord tissue. The 3-repeated measures analysis of variance (ANOVA) was used as generalised linear modelling to examine overtime changes during pregnancy in relation to the intervention. The 3-repeated measures ANOVA was also used to produce *p* for trend over time within each group. Pearson's correlation coefficient (r) was used to correlate maternal PC and PBMC fatty acid composition. A *p*-value of 0.05 was considered statistically significant for all tests.

4.3 Results

4.3.1 Fatty acid composition of lipid classes in maternal plasma during pregnancy

4.3.1.1 Fatty acid composition of plasma phospholipids (PC)

Table 4.2 describes the maternal plasma PC fatty acid composition expressed as percentages (%) of each fatty acid, for the two groups during pregnancy (20, 34, and 38 weeks gestation). Repeated measures ANOVA identified significant effects of time on the percentages of a number of fatty acids and significant effects of group (i.e. salmon vs. control) on the percentages of trans oleic acid (18:1n-9), α-linolenic acid (18:3n-3), EPA, DPA, DHA, total n-3 PUFA, dihomo-γ-linolenic acid (DGLA; 20:3n-6), ARA and total n-6 PUFA. There were significant time x group interactions for EPA, DHA, total n-3 PUFA, DGLA, ARA and total n-6 PUFA. These were explored further by pair-wise comparisons between groups at each time point. At 20 weeks gestation (baseline, before intervention) the two groups differed significantly in LC n-3 PUFA content: the salmon group had significantly higher percentages of EPA, DHA, and total n-3 PUFA compared to the control group. Also, the salmon group had lower ARA percentages at baseline compared to the control group. At 34 and 38 weeks gestation (during intervention) the salmon group continued to exhibit significantly higher percentages of EPA, DHA and total n-3 PUFA (and also DPA), and lower percentages of ARA (and also DGLA and total n-6 PUFA). The percentages of EPA, DHA, and total n-3 PUFA decreased during pregnancy in the control group (p for trend = 0.029, 0.008, 0.002), whereas they increased in the salmon group (all p for trend < 0.001) (Figure 4.13). The changes in % EPA and DHA in plasma PC were significantly different between groups ($p < 0.001$ for both) (Table 4.3). Also, plasma PC % ARA decreased significantly in both groups (both p for trend < 0.001), but the decrease was greater in the salmon group (Table 4.3). The decrease in total % n-6 PUFA was greater in the salmon group compared to the control group.

Table 4.4 describes the absolute concentrations (μg/mL plasma) of plasma PC fatty acids for both groups during pregnancy. Repeated measures ANOVA identified significant effects of time on the concentrations of all fatty acids detected in PC and in total plasma PC fatty acid. There were significant effects of group (i.e. salmon vs. control) on the concentrations of EPA, DPA, DHA, and total n-3 PUFA, but not on any other fatty acid. There were significant time x group interactions for EPA and DGLA only. These were explored further by pair-wise comparisons between groups at each time point. There were no significant differences in plasma PC fatty acid concentrations between the two groups at baseline (20 weeks). At 34 and 38 weeks gestation, the salmon group had higher absolute

concentrations of EPA, DPA, DHA, and total n-3 PUFA, and a lower concentration of ARA compared to the control group. Total n-6 PUFA concentration was significantly lower in the salmon group compared to the control group at 34 weeks. The concentrations of all fatty acids increased from week 20 to week 34 in both groups and then declined at week 38 (Table 4.4). The changes in EPA concentration in plasma PC from week 20 to week 34 were significantly different between groups (p = 0.002 for both) (Table 4.5). There were no significant differences in changes in concentrations of other fatty acids between the groups (Table 4.5). Finally, Table 4.4 includes the ratios of fatty acids of interest in the plasma PC fraction. ARA/EPA, ARA/DHA, ARA/(EPA+DHA), and n-6 PUFA/n-3 PUFA were affected by time and group and there was a significant time x group interaction. All four ratios were significantly lower in the salmon group compared to the control group at baseline, and at 34 and 38 weeks gestation. The ARA/DHA and ARA/(DHA+EPA) ratios remained almost unchanged in the control group but they decreased significantly in the salmon group (p for trend < 0.001 for both ratios).

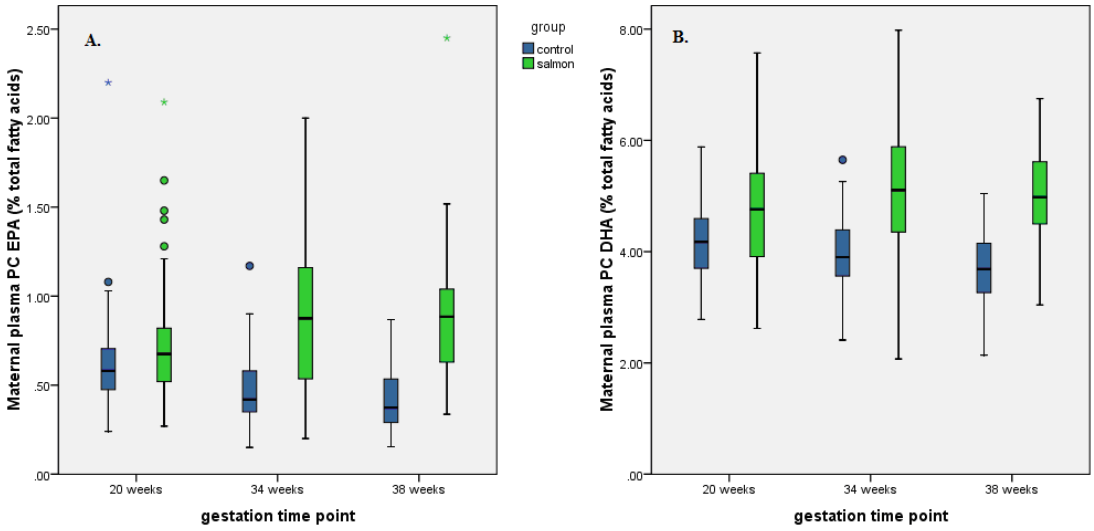


Figure 4.13 Maternal plasma PC EPA (A) and DHA (B) expressed as percentage of total fatty acids during pregnancy in both groups
p < 0.05; for exact values see text and Table 4.2

Table 4.2 Maternal plasma phospholipid (PC) fatty acid content (%) during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p**	Control (n = 55)	Salmon (n = 56)	*p**	Control (n = 41)	Salmon (n = 47)	*p**	Group	Time	Time × group
14:0	0.40 (±0.12	0.40 (±0.11)	0.738	0.40 (±0.09)	0.40 (±0.09)	0.669	0.39 (±0.11)	0.40 (±0.10)	0.595	0.555	0.590	0.939
16:0	33.01 (±1.74)	32.84 (±1.40)	0.572	34.11 (±1.08)	34.18 (±1.71)	0.794	34.47 (±0.85)	34.30 (±0.98)	0.393	0.640	**<0.001**	0.637
18:0	10.83 (±0.99)	10.67 (±1.02)	0.379	9.75 (±0.79)	9.69 (±0.80)	0.670	9.83 (±0.86)	9.72 (±0.81)	0.551	0.728	**<0.001**	0.697
20:0	0.10 (±0.10)	0.10 (±0.03)	0.698	0.09 (±0.08)	0.11 (±0.11)	0.414	0.05 (±0.02)	0.05 (±0.03)	0.664	0.934	**<0.001**	0.569
SFA	44.33 (±2.02)	44.01 (±1.68)	0.342	44.36 (±0.73)	44.39 (±1.46)	0.894	44.74 (±0.71)	44.48 (±0.70)	0.087	0.431	0.078	0.613
16:1n-7	0.86 (±0.30)	0.78 (±0.21)	0.099	0.86 (±0.21)	0.82 (±0.24)	0.301	0.86 (±0.24)	0.83 (±0.27)	0.558	0.300	0.097	0.248
18:1n-9	11.21 (±1.54)	11.12 (±1.41)	0.737	11.93 (±1.24)	11.69 (±1.33)	0.317	12.07 (±1.18)	12.00 (±1.22)	0.777	0.530	**<0.001**	0.636
18:1n-9 Trans	1.48 (±0.24)	1.46 (±0.24)	0.676	1.40 (±0.17)	1.31 (±0.16)	**0.005**	1.45 (±0.18)	1.35 (±0.17)	**0.008**	**0.010**	**<0.001**	0.651
20:1n-9	0.21 (±0.09)	0.22 (±0.15)	0.537	0.20 (±0.04)	0.21 (±0.08)	0.348	0.16 (±0.03)	0.16 (±0.03)	0.520	0.688	**<0.001**	0.734
MUFA	13.76 (1.75)	13.59 (1.59)	0.570	14.39 (±1.34)	14.02 (±1.45)	0.168	14.55 (±1.27)	14.34 (±1.36)	0.459	0.273	**<0.001**	0.792
18:3n-3	0.32 (±0.11)	0.35 (±0.12)	0.138	0.37 (±0.11)	0.43 (±0.15)	**0.020**	0.37 (±0.12)	0.43 (±0.16)	**0.041**	**0.038**	**<0.001**	0.217
20:5n-3 (EPA)	0.62 (±0.27)	0.74 (±0.33)	**0.028**	0.46 (±0.19)	0.89 (±0.42)	**<0.001**	0.42 (±0.18)	0.89 (±0.39)	**<0.001**	**<0.001**	0.758	**<0.001**
22:5n-3 (DPA)	0.67 (±0.18)	0.72 (±0.17)	0.189	0.58 (±0.13)	0.70 (±0.17)	**<0.001**	0.53 (±0.14)	0.62 (±0.14)	**0.003**	**0.002**	**<0.001**	0.063
22:6n-3 (DHA)	4.19 (±0.71)	4.71 (±0.98)	**0.001**	3.99 (±0.66)	5.12 (±1.14)	**<0.001**	3.71 (±0.67)	5.02 (±0.89)	**<0.001**	**<0.001**	**0.003**	**<0.001**
Total n-3	5.79 (±0.96)	6.51 (±1.21)	**<0.001**	5.41 (±0.83)	7.14 (±1.55)	**<0.001**	5.03 (±0.90)	6.97 (±1.25)	**<0.001**	**<0.001**	**0.005**	**<0.001**
18:2n-6	21.45 (±2.51)	22.06 (±2.45)	0.175	22.24 (±2.20)	22.68 (±2.31)	0.315	22.68 (±2.40)	22.74 (±2.22)	0.903	0.666	**<0.001**	0.877
18:3n-6	0.10 (±0.08)	0.11 (±0.15)	0.899	0.10 (±0.10)	0.10 (±0.12)	0.790	0.06 (±0.02)	0.05 (±0.02)	0.402	0.790	**0.003**	0.117
20:2n-6	0.49 (±0.15)	0.49 (±0.11)	0.965	0.45 (±0.09)	0.47 (±0.09)	0.173	0.39 (±0.08)	0.41 (±0.08)	0.359	0.650	**<0.001**	0.098
20:3n-6	4.39 (±0.71)	4.20 (±0.77)	0.149	4.35 (±0.72)	3.70 (±0.57)	**<0.001**	4.17 (±0.62)	3.65 (±0.57)	**<0.001**	**<0.001**	**<0.001**	**0.011**
20:4n-6 (ARA)	9.67 (±1.48)	9.03 (±1.77)	**0.032**	8.70 (±1.34)	7.49 (±1.37)	**<0.001**	8.38 (±1.23)	7.36 (±1.30)	**<0.001**	**0.002**	**<0.001**	**0.008**
Total n-6	36.11 (±2.30)	35.89 (±2.09)	0.576	35.84 (±1.53)	34.45 (±1.70)	**<0.001**	35.68 (±1.74)	34.22 (±1.90)	**<0.001**	**<0.001**	**<0.001**	**0.048**
PUFA	41.91 (±2.37)	42.40 (±1.93)	0.210	41.25 (±1.56)	41.59 (±1.77)	0.287	40.71 (±1.45)	41.19 (±1.51)	0.142	0.153	**<0.001**	0.858

**p*-values from independent samples t-test

Values are mean (± standard deviation) percentage levels (%) of total fatty acids in PC

Table 4.3 Changes of percentage phospholipid levels (% PC fatty acids) for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	*p**	Control (n = 41)	Salmon (n = 47)	*p**
20:5n-3 (EPA)	-0.16 (±0.30)	0.15 (±0.45)	**<0.001**	-0.17 (±0.16)	0.13 (±0.40)	**<0.001**
22:6n-3 (DHA)	-0.17 (±0.50)	0.38 (±0.93)	**<0.001**	-0.41 (±0.49)	0.13 (±0.69)	**<0.001**
20:4n-6 (ARA)	-1.09 (±0.94)	-1.54 (±1.36)	**0.043**	-1.30 (±0.81)	-1.86 (±1.08)	**0.008**

**p*-values from independent samples t-test

Values are mean (± standard deviation) of changes in percentage levels (%)

Table 4.4 Absolute concentration of maternal plasma phospholipid (PC) fatty acids during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p**	Control (n = 55)	Salmon (n = 56)	*p**	Control (n = 41)	Salmon (n = 47)	*p**	Group	Time	Time × group
14:0	10.9 (±5.8)	10.2 (±5.1)	0.465	13.8 (±5.1)	13.1 (±5.1)	0.464	8.0 (±3.0)	8.2 (±2.9)	0.754	0.921	**<0.001**	0.507
16:0	829.1 (±340.8)	813.9 (±331.6)	0.804	1163.7 (±287.9)	1092.8 (±271.9)	0.187	701.0 (±134.5)	702.0 (±162.4)	0.973	0.935	**<0.001**	0.263
18:0	271.3 (±111.9)	264.1 (±109.4)	0.718	331.3 (±81.3)	310.3 (±81.2)	0.177	199.8 (±42.1)	197.9 (±43.1)	0.838	0.990	**<0.001**	0.218
20:0	5.3 (±4.9)	4.6 (±3.2)	0.347	3.1 (±2.2)	3.6 (±4.8)	0.538	1.1 (±0.4)	1.1 (±0.5)	0.801	0.467	**<0.001**	0.250
SFA	1116.73 (±455.46)	1092.80 (±441.61)	0.770	1511.89 (±367.89)	1419.69 (±351.04)	0.182	909.82 (±174.49)	909.25 (±204.19)	0.989	0.946	**<0.001**	0.257
16:1n-7	22.0 (±13.2)	19.3 (±9.4)	0.196	30.0 (±11.9)	26.7 (±12.5)	0.160	17.8 (±6.7)	17.2 (±7.0)	0.688	0.463	**<0.001**	0.671
18:1n-9	288.2 (±127.2)	279.2 (±121.9)	0.693	408.1 (±105.4)	376.0 (±112.8)	0.126	246.4 (±57.7)	247.5 (±70.8)	0.939	0.672	**<0.001**	0.193
18:1n-9 Trans	38.5 (±16.5)	36.7 (±15.4)	0.524	47.4 (±11.5)	42.2 (±11.9)	**0.023**	29.3 (±6.0)	27.4 (±6.3)	0.145	0.189	**<0.001**	0.270
20:1n-9	5.9 (±4.0)	5.5 (±3.0)	0.599	6.7 (±2.0)	6.9 (±4.2)	0.754	3.3 (±0.6)	3.2 (±0.9)	0.779	0.922	**<0.001**	0.874
MUFA	354.60 (±155.86)	340.73 (±146.30)	0.615	492.17 (±125.55)	451.85 (±134.89)	0.108	296.78 (±67.55)	295.28 (±81.68)	0.926	0.584	**<0.001**	0.231
18:3n-3	6.3 (±6.5)	6.9 (±6.4)	0.635	12.9 (±5.5)	14.0 (±6.1)	0.309	7.6 (±2.9)	9.0 (±4.4)	0.098	0.099	**<0.001**	0.865
20:5n-3 (EPA)	16.3 (±12.7)	18.7 (±13.8)	0.312	16.1 (±9.2)	29.4 (±16.7)	**<0.001**	8.6 (±4.2)	18.4 (±10.2)	**<0.001**	**<0.001**	**<0.001**	**0.008**
22:5n-3 (DPA)	18.0 (±10.9)	18.1 (±10.0)	0.930	20.0 (±7.1)	23.0 (±8.5)	**0.046**	10.9 (±4.0)	12.7 (±3.9)	**0.037**	**0.033**	**<0.001**	0.398
22:6n-3 (DHA)	104.3 (±50.1)	115.2 (±57.9)	0.271	134.6 (±36.3)	167.0 (±60.6)	**0.001**	75.1 (±18.6)	102.1 (±26.1)	**<0.001**	**<0.001**	**<0.001**	0.398
Total n-3	144.9 (±76.24)	158.97 (±82.37)	0.332	183.61 (±52.66)	233.36 (±82.81)	**<0.001**	102.20 (±26.73)	142.15 (±39.71)	**<0.001**	**<0.001**	**<0.001**	0.279
18:2n-6	539.8 (±222.0)	541.6 (±217.3)	0.965	763.8 (±206.9)	723.8 (±177.8)	0.280	460.0 (±95.8)	462.4 (±102.3)	0.913	0.948	**<0.001**	0.212
18:3n-6	2.8 (±2.2)	2.2 (±1.7)	0.085	3.4 (±4.0)	3.4 (±5.7)	0.980	1.2 (±0.6)	1.1 (±0.6)	0.482	0.982	**0.001**	0.272
20:2n-6	13.2 (±7.6)	12.3 (±5.4)	0.449	15.4 (±5.2)	15.4 (±5.5)	0.968	8.0 (±2.2)	8.3 (±2.3)	0.524	0.959	**<0.001**	0.844
20:3n-6	112.3 (±50.0)	105.3 (±50.0)	0.438	147.8 (±43.5)	119.2 (±39.3)	**<0.001**	85.1 (±22.5)	75.5 (±24.3)	0.061	0.055	**<0.001**	**0.028**
20:4n-6 (ARA)	241.3 (±107.0)	223.1 (±106.0)	0.350	292.1 (±76.8)	242.2 (±75.5)	**0.001**	170.0 (±40.9)	150.4 (±41.2)	**0.029**	0.138	**<0.001**	0.072
Total n-6	909.40 (±368.5)	884.40 (±356.54)	0.705	1222.48 (±303.17)	1103.98 (±269.97)	**0.033**	724.22 (±136.88)	697.61 (±152.66)	0.397	0.418	**<0.001**	0.129
PUFA	1054.30 (±439.32)	1043.37 (±430.40)	0.890	1406.09 (±347.19)	1337.34 (±335.02)	0.293	826.42 (±153.62)	839.76 (±181.21)	0.714	0.772	**<0.001**	0.329
Total fatty acids	2525.63 (±1037.77)	2476.9 (±1006.53)	0.794	3410.16 (±826.58)	3208.89 (±802.16)	0.198	2033.02 (±387.39)	2044.29 (±459.63)	0.903	0.999	**<0.001**	0.278
ARA/DHA	2.43 (±0.51)	2.01 (±0.41)	**<0.001**	2.20 (±0.37)	1.52 (±0.36)	**<0.001**	2.30 (±0.38)	1.50 (±0.35)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ARA/EPA	18.21 (±7.10)	14.41 (±5.30)	**0.001**	21.29 (±8.50)	10.57 (±5.69)	**<0.001**	23.59 (±10.12)	9.89 (±4.61)	**<0.001**	**<0.001**	0.366	**<0.001**
ARA/(DHA+EPA)	2.13 (±0.48)	1.74 (±0.36)	**<0.001**	1.98 (±0.33)	1.31 (±0.34)	**<0.001**	2.07 (±0.36)	1.29 (±0.33)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
n-6/n-3	6.85 (±1.54)	6.03 (±1.45)	**0.003**	6.79 (±1.13)	5.09 (±1.56)	**<0.001**	7.36 (±1.57)	5.11 (±1.21)	**<0.001**	**<0.001**	**<0.001**	**0.001**

**p*-values from independent samples t-test

Values are mean (± standard deviation) absolute concentrations (µg fatty acid/mL plasma) in PC

Table 4.5 Changes in phospholipid (PC) fatty acid absolute concentration for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	p^*	Control (n = 41)	Salmon (n = 47)	p^*
20:5n-3 (EPA)	-0.33 (±14.85)	10.27 (±19.77)	**0.002**	-5.64 (±9.45)	-1.34 (±15.98)	0.136
22:6n-3 (DHA)	30.50 (±57.85)	47.78 (±72.56)	0.170	-20.69 (±43.40)	-19.80 (±50.12)	0.930
20:4n-6 (ARA)	49.31 (±127.14)	12.52 (±120.92)	0.123	-55.70 (±90.59)	-82.06 (±86.83)	0.170

*p-values from independent samples t-test

Values are mean (± standard deviation) of changes in absolute concentration (µg/mL)

4.3.1.2 Fatty acid composition of plasma triglycerides (TAG)

Table 4.6 describes the maternal plasma TAG fatty acid composition expressed as percentages (%) of each fatty acid, for the two groups during pregnancy (20, 34, and 38 weeks gestation). Repeated measures ANOVA identified significant effects of time and group on the percentages of a number of fatty acids. There were significant time x group interactions for EPA, DPA, DHA, and total n-3 PUFA. These were explored further by pair-wise comparisons between groups at each time point. At 20 weeks gestation (baseline, before intervention) the two groups differed significantly in LC n-3 PUFA content: the salmon group had significantly higher percentages of EPA, DHA, and total n-3 PUFA compared to the control group. At 34 and 38 weeks gestation (during intervention) the salmon group continued to exhibit significantly higher percentages of EPA, DHA and total n-3 PUFA (and also DPA). The percentages of EPA, DHA, and total n-3 PUFA decreased during pregnancy in the control group (p for trend < 0.001, 0.001, 0.041), whereas the percentage did not change for EPA (p for trend = 0.281) and they increased significantly for DHA and total n-3 PUFA in the salmon group (p for trend < 0.001, 0.007). The changes in % EPA and DHA in plasma TAG were significantly different between groups (p < 0.001 for both) (Table 4.7). Also, plasma TAG % ARA decreased significantly in both groups (both p for trend < 0.001), with little difference between them (Table 4.7).

Table 4.8 describes the absolute concentration (µg/mL plasma) of plasma TAG fatty acids for both groups during pregnancy. Repeated measures ANOVA identified significant effects of time on the concentrations of all fatty acids (except ARA and DGLA) detected in TAG and in total plasma TAG fatty acid. There were significant effects of group (i.e. salmon vs. control) on the concentrations of EPA, DPA, DHA, and total n-3 PUFA, but not on most other fatty acids. There were significant time x group interactions for stearic acid (18:0), EPA, DHA, and total n-3 PUFA. These were explored further by pair-wise comparisons between groups at each time point. The concentration of ARA in plasma TAG was significantly lower in the salmon group at baseline. At 34 and 38 weeks gestation, the salmon group had higher absolute concentrations of EPA and DHA, and at 34 weeks lower concentrations of linoleic acid (18:3n-6), γ-linolenic acid (GLA;18:3n-6), DGLA, ARA and total n-6 PUFA compared to the control group. The concentrations of n-3 PUFA increased from week 20 to week 34 and from week 34 to week 38 in both groups (Table 4.8), but the increases were greater in the salmon group. The increases in EPA and DHA concentration in plasma TAG from week 20 to week 34 and from week 20 to week 38 were significantly different between groups ($p \leq 0.002$ for all) (Table 4.9). There were no significant differences in changes in concentration of ARA in plasma TAG between the

groups (Table 4.9). Finally, Table 4.8 includes the ratios of fatty acids of interest in the plasma TAG fraction. ARA/EPA, ARA/DHA, ARA/(EPA+DHA), and n-6 PUFA/n-3 PUFA were affected by time and group, but there was a significant time x group interaction only for ARA/EPA. All four ratios were significantly lower in the salmon group compared to the control group at baseline, and at 34 and 38 weeks gestation and all four ratios declined to a greater extent in the salmon group.

Table 4.6 Maternal plasma triglyceride (TAG) fatty acid content (%) during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p**	Control (n = 55)	Salmon (n = 56)	*p**	Control (n = 41)	Salmon (n = 47)	*p**	Group	Time	Time × group
14:0	2.23 (±0.84)	2.30 (±0.79)	0.892	2.27 (±0.63)	2.32 (±0.63)	0.687	2.27 (±0.67)	2.32 (±0.74)	0.720	0.907	0.915	0.698
16:0	28.38 (±3.22)	28.25 (±3.60)	0.837	31.01 (±2.54)	31.15 (±2.97)	0.792	32.11 (±2.83)	32.14 (±4.67)	0.965	0.742	**<0.001**	0.917
18:0	2.54 (±0.50)	2.79 (±0.68)	**0.022**	2.84 (±0.46)	2.95 (±0.59)	0.263	2.80 (±0.59)	3.16 (±0.82)	**0.020**	**0.001**	**<0.001**	0.262
20:0	0.05 (±0.03)	0.05 (±0.03)	0.595	0.15 (±0.31)	0.24 (±0.49)	0.223	0.05 (±0.03)	0.06 (±0.04)	**0.033**	0.231	**0.002**	0.334
SFA	33.29 (±4.01)	33.39 (±4.44)	0.890	36.27 (±3.07)	36.67 (±3.76)	0.546	37.22 (±3.53)	37.69 (±5.43)	0.635	0.475	**<0.001**	0.985
16:1n-7	4.08 (±1.35)	3.64 (±1.11)	0.055	3.91 (±0.99)	3.66 (±1.02)	0.195	3.99 (±1.13)	3.67 (±1.19)	0.206	0.116	0.433	0.498
18:1n-9	38.55 (±3.36)	38.40 (±3.12)	0.800	38.59 (±3.08)	37.35 (±2.91)	**0.033**	38.11 (±3.83)	36.13 (±4.24)	**0.025**	**0.037**	**0.003**	0.118
18:1n-9 Trans	2.64 (±0.46)	2.54 (±0.44)	0.217	2.60 (±0.38)	2.37 (±0.36)	**0.002**	2.49 (±0.40)	2.14 (±0.41)	**<0.001**	**<0.001**	**<0.001**	0.191
20:1n-9	0.46 (±0.11)	0.44 (±0.09)	0.372	0.52 (±0.10)	0.50 (±0.09)	0.232	0.45 (±0.11)	0.39 (±0.10)	**0.014**	**0.010**	**<0.001**	0.486
MUFA	45.73 (±3.51)	45.02 (±3.09)	0.245	45.62 (±3.07)	43.88 (±2.99)	**0.003**	45.04 (±3.54)	42.34 (±4.09)	**0.001**	**0.001**	**0.001**	0.109
18:3n-3	1.15 (±0.39)	1.25 (±0.38)	0.176	1.04 (±0.47)	1.13 (±0.57)	0.397	1.15 (±0.44)	1.48 (±0.69)	**0.010**	**0.033**	**0.034**	0.117
20:5n-3 (EPA)	0.20 (±0.09)	0.24 (±0.15)	**0.045**	0.14 (±0.06)	0.28 (±0.15)	**<0.001**	0.11 (±0.06)	0.29 (±0.17)	**<0.001**	**<0.001**	0.172	**<0.001**
22:5n-3 (DPA)	0.19 (±0.07)	0.20 (±0.07)	0.542	0.17 (±0.06)	0.23 (±0.10)	**<0.001**	0.16 (±0.09)	0.25 (±0.14)	**0.001**	**<0.001**	0.452	**0.005**
22:6n-3 (DHA)	0.78 (±0.24)	0.93 (±0.41)	**0.017**	0.67 (±0.22)	1.27 (±0.68)	**<0.001**	0.65 (±0.28)	1.50 (±0.81)	**<0.001**	**<0.001**	**0.009**	**<0.001**
Total n-3	2.32 (±0.59)	2.62 (±0.78)	**0.019**	2.01 (±0.54)	2.92 (±1.27)	**<0.001**	2.07 (±0.73)	3.52 (±1.61)	**<0.001**	**<0.001**	0.073	**0.002**
18:2n-6	15.93 (±3.71)	16.44 (±3.39)	0.435	14.13 (±2.72)	14.66 (±2.93)	0.330	13.90 (±2.52)	14.59 (±3.17)	0.265	0.408	**<0.001**	0.810
18:3n-6	0.27 (±0.13)	0.25 (±0.13)	0.303	0.15 (±0.06)	0.14 (±0.05)	0.339	0.17 (±0.10)	0.20 (±0.13)	0.216	0.921	**<0.001**	0.094
20:2n-6	0.25 (±0.10)	0.25 (±0.06)	0.807	0.25 (±0.07)	0.25 (±0.07)	0.828	0.23 (±0.07)	0.22 (±0.05)	0.411	0.369	**0.001**	0.898
20:3n-6	0.40 (±0.12)	0.38 (±0.09)	0.355	0.32 (±0.09)	0.29 (±0.06)	0.074	0.28 (±0.10)	0.28 (±0.16)	0.831	0.271	**<0.001**	0.226
20:4n-6 (ARA)	1.81 (±0.58)	1.65 (±0.52)	0.110	1.24 (±0.40)	1.19 (±0.33)	0.414	1.09 (±0.34)	1.16 (±0.47)	0.480	0.644	**<0.001**	0.164
Total n-6	18.66 (±4.12)	18.96 (±3.62)	0.672	16.09 (±2.87)	16.53 (±3.05)	0.442	15.67 (±2.74)	16.45 (±3.54)	0.255	0.515	**<0.001**	0.642
PUFA	20.98 (±4.34)	21.58 (±4.06)	0.436	18.11 (±3.11)	19.45 (±3.67)	**0.042**	17.74 (±3.15)	19.97 (±4.45)	**0.009**	**0.039**	**<0.001**	0.165

**p*-values from independent samples t-test

Values are mean (± standard deviation) percentage levels (%) of total fatty acids in triglycerides

Table 4.7 Changes of percentage triglyceride levels (%TAG) for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	*p**	Control (n = 41)	Salmon (n = 47)	*p**
20:5n-3 (EPA)	-0.06 (±0.10)	0.03 (±0.16)	**<0.001**	-0.07 (±0.07)	0.02 (±0.14)	**<0.001**
22:6n-3 (DHA)	-0.12 (±0.19)	0.31 (±0.50)	**<0.001**	-0.12 (±0.23)	0.50 (±0.68)	**<0.001**
20:4n-6 (ARA)	-0.57 (±0.46)	-0.49 (±0.43)	0.389	-0.73 (±0.51)	-0.54 (±0.46)	0.079

**p*-values from independent samples t-test

Values are mean (± standard deviation) of changes in percentage levels (%)

Table 4.8 Absolute concentration of maternal plasma triglyceride (TAG) fatty acids during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	Group	Time	Time × group
14:0	25.4 (±15.3)	22.0 (±14.3)	0.219	39.8 (±20.8)	33.5 (±15.7)	0.074	38.2 (±18.6)	40.5 (±28.1)	0.664	0.610	**<0.001**	0.228
16:0	307.1 (±160.0)	273.2 (±158.5)	0.244	548.4 (±270.6)	447.7 (±177.8)	**0.023**	530.8 (±219.9)	564.3 (±318.8)	0.573	0.709	**<0.001**	0.109
18:0	26.9 (±13.1)	26.2 (±15.1)	0.801	49.6 (±21.7)	41.6 (±15.3)	**0.026**	47.1 (±22.8)	56.6 (±39.4)	0.176	0.643	**<0.001**	**0.038**
20:0	0.5 (±0.3)	0.4 (±0.2)	0.221	2.1 (±4.1)	3.2 (±6.8)	0.324	0.9 (±0.8)	1.3 (±1.3)	0.103	0.314	**0.002**	0.449
SFA	359.80 (±185.85)	321.84 (±186.12)	0.264	640.05 (±308.94)	525.93 (±205.47)	**0.025**	616.91 (±257.63)	662.65 (±380.72)	0.517	0.758	**<0.001**	0.094
16:1n-7	45.7 (±33.3)	35.6 (±20.7)	**0.047**	70.2 (±44.6)	52.8 (±26.0)	**0.014**	66.3 (±33.2)	65.6 (±43.0)	0.934	0.273	**<0.001**	0.415
18:1n-9	404.7 (±172.5)	360.3 (±170.5)	0.157	670.0 (±268.0)	531.7 (±195.0)	**0.003**	610.6 (±227.1)	621.6 (±304.9)	0.850	0.293	**<0.001**	0.058
18:1n-9 Trans	28.0 (±13.8)	23.7 (±10.8)	0.055	45.2 (±20.0)	33.6 (±12.2)	**<0.001**	40.0 (±16.3)	36.0 (±17.2)	0.273	**0.021**	**<0.001**	0.189
20:1n-9	4.8 (±2.2)	4.2 (±2.1)	0.080	9.0 (±3.6)	7.0 (±2.6)	**0.001**	7.3 (±3.6)	6.4 (±2.6)	0.180	**0.017**	**<0.001**	0.174
MUFA	483.26 (±215.17)	423.72 (±200.26)	0.118	794.37 (±328.74)	625.10 (±227.81)	**0.002**	724.16 (±269.29)	729.65 (±357.22)	0.936	0.241	**<0.001**	0.074
18:3n-3	12.0 (±6.0)	11.6 (±6.1)	0.707	18.4 (±11.0)	16.2 (±10.2)	0.278	19.9 (±13.6)	27.4 (±25.1)	0.094	0.286	**<0.001**	0.076
20:5n-3 (EPA)	2.1 (±1.4)	2.1 (±1.3)	0.713	2.2 (±1.0)	3.8 (±2.0)	**<0.001**	2.1 (±1.8)	4.8 (±4.3)	**<0.001**	**<0.001**	**<0.001**	**0.004**
22:5n-3 (DPA)	2.0 (±1.3)	1.8 (±1.1)	0.408	2.8 (±1.5)	3.2 (±1.7)	0.214	2.9 (±2.5)	4.8 (±4.7)	**0.027**	**0.011**	**<0.001**	0.052
22:6n-3 (DHA)	8.1 (±4.2)	8.4 (±4.7)	0.728	11.1 (±4.5)	17.8 (±11.0)	**<0.001**	11.9 (±8.7)	27.9 (±27.7)	**0.001**	**<0.001**	**<0.001**	**0.004**
Total n-3	24.20 (±11.18)	23.98 (±11.62)	0.916	34.52 (±14.91)	40.96 (±22.73)	0.082	36.91 (±24.97)	64.85 (±59.49)	0.006	0.002	**<0.001**	**0.020**
18:2n-6	163.5 (±70.8)	149.4 (±65.7)	0.255	243.8 (±102.5)	206.4 (±79.0)	**0.034**	229.8 (±117.0)	256.0 (±153.1)	0.375	0.727	**<0.001**	0.073
18:3n-6	2.8 (±1.7)	2.3 (±1.6)	0.081	2.5 (±1.4)	1.9 (±0.9)	**0.011**	3.1 (±3.3)	3.9 (±3.9)	0.320	0.980	**0.001**	0.155
20:2n-6	2.6 (±1.7)	2.2 (±1.1)	0.124	4.4 (±2.1)	3.6 (±1.6)	**0.039**	4.0 (±2.6)	3.8 (±2.3)	0.790	0.163	**<0.001**	0.375
20:3n-6	4.2 (±2.2)	3.6 (±1.9)	0.106	5.4 (±2.2)	4.1 (±1.7)	**0.001**	4.8 (±3.6)	5.5 (±5.5)	0.514	0.495	0.011	0.109
20:4n-6 (ARA)	18.2 (±7.4)	14.8 (±6.9)	**0.012**	20.2 (±6.9)	16.3 (±5.9)	**0.002**	18.5 (±11.6)	20.5 (±15.7)	0.486	0.407	0.064	0.100
Total n-6	191.33 (±79.90)	172.28 (±74.10)	0.176	276.26 (±110.58)	232.34 (±85.73)	**0.022**	260.21 (±135.15)	289.79 (±176.13)	0.385	0.675	**<0.001**	0.071
PUFA	215.52 (±88.53)	196.26 (±84.17)	0.222	310.78 (±122.73)	273.30 (±100.73)	0.083	297.12 (±156.50)	354.64 (±227.79)	0.177	0.807	**<0.001**	0.052
Total fatty acids	1058.58 (±466.10)	941.82 (±454.04)	0.165	1745.20 (±733.43)	1424.33 (±504.63)	**0.009**	1638.19 (±658.13)	1746.93 (±915.27)	0.529	0.562	**<0.001**	0.060
ARA/DHA	2.41 (±0.73)	1.94 (±0.62)	**<0.001**	1.92 (±0.51)	1.10 (±0.47)	**<0.001**	1.76 (±0.55)	0.95 (±0.48)	**<0.001**	**<0.001**	**<0.001**	0.096
ARA/EPA	10.50 (±4.05)	8.15 (±3.16)	**0.001**	10.11 (±3.79)	5.14 (±2.45)	**<0.001**	10.87 (±4.55)	5.66 (±4.06)	**<0.001**	**<0.001**	**0.001**	**0.027**
ARA/(DHA+EPA)	1.93 (±0.56)	1.54 (±0.48)	**<0.001**	1.60 (±0.41)	0.90 (±0.38)	**<0.001**	1.50 (±0.47)	0.81 (±0.42)	**<0.001**	**<0.001**	**<0.001**	0.069
n-6/n-3	8.43 (±2.47)	7.62 (±1.81)	**0.042**	8.45 (±2.33)	6.62 (±2.97)	**0.001**	8.01 (±2.87)	5.71 (±2.39)	**<0.001**	**<0.001**	**<0.001**	0.134

**p*-Values from independent samples t-test

Values are mean (± standard deviation) absolute concentrations (µg fatty acid/mL plasma) in TAG

Table 4.9 Changes in triglyceride (TAG) fatty acid absolute concentration for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	*p**	Control (n = 41)	Salmon (n = 47)	*p**
20:5n-3 (EPA)	0.07 (±1.60)	1.64 (±2.21)	**<0.001**	0.26 (±1.88)	2.54 (±4.11)	**0.002**
22:6n-3 (DHA)	2.78 (±5.19)	9.21 (±9.94)	**<0.001**	4.23 (±7.63)	18.93 (±26.38)	**0.001**
20:4n-6 (ARA)	1.68 (±8.63)	1.43 (±7.15)	0.867	0.69 (±10.10)	5.63 (±14.72)	0.074

* *p*-Values from independent samples t-test

Values are mean (± standard deviation) of changes in absolute concentration (µg/mL)

4.3.1.3 Fatty acid composition of plasma cholesteryl esters (CE)

Table 4.10 describes the maternal plasma CE fatty acid composition expressed as percentages (%) of each fatty acid, for the two groups during pregnancy (20, 34, and 38 weeks gestation). Repeated measures ANOVA identified a significant effect of time on percentages of most fatty acids and a significant effect of group on the percentages of EPA, DHA, total n-3 PUFA, DGLA and ARA. There were significant time x group interactions for EPA, DHA, and total n-3 PUFA. These were explored further by pair-wise comparisons between groups at each time point. At 20 weeks gestation the salmon group had significantly lower percentages of DGLA and ARA compared to the control group. At 34 and 38 weeks gestation (during intervention) the salmon group exhibited significantly higher percentages of EPA, DHA and total n-3 PUFA and significantly lower percentages of DGLA and ARA. The percentages of EPA and DHA decreased during pregnancy in the control group (p for trend < 0.001, 0.039), whereas the percentage of EPA increased significantly (p for trend = 0.030) and that of DHA did not change in the salmon group (p for trend = 0.220). The changes in % EPA and DHA in plasma CE were significantly different between groups (Table 4.11). Also, plasma CE % ARA decreased significantly in both groups (both p for trend < 0.001), with little difference between them (Table 4.11).

Table 4.12 describes the absolute concentration (μg/mL plasma) of plasma CE fatty acids for both groups during pregnancy. Repeated measures ANOVA identified significant effects of time on the concentrations of almost all fatty acids detected in CE and in total plasma CE fatty acid. There were significant effects of group (i.e. salmon vs. control) on the concentrations of EPA, DHA, total n-3 PUFA, DGLA and ARA. There were significant time x group interactions for EPA, DPA, DHA, total n-3 PUFA and ARA. These were explored further by pair-wise comparisons between groups at each time point. The concentration of DGLA in plasma CE was significantly lower in the salmon group at baseline. At 34 and 38 weeks gestation, the salmon group had higher absolute concentrations of EPA and DHA, and lower concentrations of DGLA and ARA compared to control group. The concentration of EPA decreased and that of DHA increased over time in the control group (p for trend = 0.262, < 0.001). In the salmon group the concentrations of both EPA and DHA increased (both p for trend < 0.001). The changes in EPA concentration in plasma CE from week 20 to week 34, and in both EPA and DHA from week 20 to week 38 were significantly different between groups ($p \leq 0.015$ for all) (Table 4.13). Finally, Table 4.12 includes the ratios of fatty acids of interest in the plasma

CE fraction. ARA/EPA, ARA/DHA, ARA/(EPA+DHA), and n-6 PUFA/n-3 PUFA were affected by time and group, and there were significant time x group interactions for all four ratios. These ratios changed little in the control group but decreased significantly over time in the salmon group (all ratios p for trend < 0.001 for the salmon group).

Table 4.10 Maternal plasma cholesteryl ester (CE) fatty acid content (%) during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	Group	Time	Time × group
14:0	0.80 (±0.21)	0.83 (±0.20)	0.374	0.74 (±0.19)	0.76 (±0.20)	0.624	0.94 (±0.45)	1.19 (±0.68)	0.057	0.074	**<0.001**	0.085
16:0	12.17 (±0.76)	11.93 (±0.76)	0.091	12.20 (±0.71)	12.11 (±0.90)	0.538	14.39 (±5.52)	16.05 (±6.29)	0.196	0.221	**<0.001**	0.160
18:0	0.75 (±0.26)	0.78 (±0.41)	0.618	0.62 (±0.33)	0.59 (±0.28)	0.656	0.85 (±0.61)	1.07 (±0.77)	0.145	0.260	**<0.001**	0.166
20:0	0.14 (±0.07)	0.15 (±0.07)	0.514	0.77 (±0.28)	0.84 (±0.36)	0.317	0.03 (±0.03)	0.04 (±0.04)	0.052	0.136	**<0.001**	0.360
SFA	13.86 (±0.90)	13.70 (±1.05)	0.360	14.34 (±1.02)	14.30 (±1.25)	0.852	16.21 (±6.50)	18.34 (±7.65)	0.166	0.159	**<0.001**	0.155
16:1n-7	3.15 (±1.28)	3.18 (±1.35)	0.876	4.01 (±1.41)	3.57 (±1.43)	0.109	4.12 (±1.18)	4.05 (±1.57)	0.829	0.651	**<0.001**	0.207
18:1n-9	20.74 (±2.48)	20.80 (±2.01)	0.873	21.09 (±2.23)	21.68 (±1.77)	0.568	23.72 (±4.00)	24.98 (±5.86)	0.250	0.344	**<0.001**	0.263
18:1n-9 Trans	1.44 (±0.25)	1.42 (±0.23)	0.653	0.98 (±0.19)	0.98 (±0.21)	0.859	1.25 (±0.34)	1.25 (±0.31)	0.958	0.962	**<0.001**	0.759
20:1n-9	0.18 (±0.12)	0.17 (±0.14)	0.633	0.11 (±0.07)	0.10 (±0.07)	0.642	0.10 (±0.11)	0.14 (±0.14)	0.193	0.512	**<0.001**	0.411
MUFA	25.51 (3.30)	25.58 (3.03)	0.901	26.99 (3.12)	26.33 (2.57)	0.231	29.19 (4.56)	30.42 (6.99)	0.339	0.557	**<0.001**	0.273
18:3n-3	0.82 (±0.24)	0.89 (±0.24)	0.126	0.44 (±0.40)	0.44 (±0.43)	0.993	0.93 (±0.25)	1.09 (±0.29)	**0.010**	0.148	**<0.001**	0.194
20:5n-3 (EPA)	0.53 (±0.25)	0.61 (±0.26)	0.067	0.42 (±0.15)	0.72 (±0.28)	**<0.001**	0.38 (±0.19)	0.68 (±0.32)	**<0.001**	**<0.001**	0.153	**0.001**
22:5n-3 (DPA)	0.24 (±0.18)	0.20 (±0.15)	0.249	0.14 (±0.06)	0.14 (±0.07)	0.928	0.06 (±0.05)	0.09 (±0.08)	**0.011**	0.713	**<0.001**	0.267
22:6n-3 (DHA)	0.68 (±0.15)	0.73 (±0.24)	0.158	0.60 (±0.13)	0.76 (±0.19)	**<0.001**	0.61 (±0.19)	0.84 (±0.22)	**<0.001**	**<0.001**	0.346	**0.040**
Total n-3	2.26 (±0.57)	2.43 (±0.54)	0.093	1.60 (±0.46)	2.07 (±0.59)	**<0.001**	1.98 (±0.48)	2.70 (±0.57)	**<0.001**	**<0.001**	**<0.001**	**0.007**
18:2n-6	49.71 (±4.23)	50.35 (±3.88)	0.387	49.40 (±4.11)	50.64 (±3.54)	0.095	45.64 (±9.26)	42.48 (±12.65)	0.190	0.395	**<0.001**	0.137
18:3n-6	0.70 (±0.24)	0.63 (±0.26)	0.128	0.46 (±0.40)	0.45 (±0.38)	0.877	0.58 (±0.24)	0.52 (±0.30)	0.285	0.405	**<0.001**	0.714
20:2n-6	0.12 (±0.13)	0.10 (±0.08)	0.166	0.07 (±0.03)	0.08 (±0.06)	0.929	0.07 (±0.06)	0.19 (±0.75)	0.306	0.405	0.406	0.285
20:3n-6	1.03 (±0.18)	0.94 (±0.19)	**0.009**	0.93 (±0.18)	0.81 (±0.25)	**0.003**	0.86 (±0.21)	0.73 (±0.26)	**0.013**	0.001	**<0.001**	0.869
20:4n-6 (ARA)	6.81 (±1.24)	6.27 (±1.42)	**0.029**	6.19 (±1.15)	5.33 (±1.10)	**<0.001**	5.48 (±1.81)	4.63 (±1.55)	**0.020**	0.013	**<0.001**	0.196
Total n-6	58.37 (±3.79)	58.29 (±3.53)	0.905	57.07 (±3.49)	57.31 (±3.06)	0.703	52.62 (±10.63)	48.54 (±13.61)	0.124	0.122	**<0.001**	0.152
PUFA	60.63 (±3.70)	60.72 (±3.40)	0.888	58.67 (±3.51)	59.37 (±3.08)	0.270	54.60 (±10.64)	51.24 (±13.53)	0.203	0.274	**<0.001**	0.182

**p*-values from independent samples t-test

Values are mean (± standard deviation) percentage levels (%) of total fatty acids in CE

Table 4.11 Changes of percentage cholesteryl ester levels (%CE) for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
20:5n-3 (EPA)	-0.11 (±0.24)	0.11 (±0.30)	**<0.001**	-0.11 (±0.18)	0.04 (±0.35)	**0.014**
22:6n-3 (DHA)	-0.07 (±0.17)	0.01 (±0.25)	**0.040**	-0.06 (±0.20)	0.07 (±0.26)	**0.013**
20:4n-6 (ARA)	-0.60 (±0.77)	-1.00 (±0.77)	**0.009**	-0.1.26 (±1.46)	-1.74 (±1.67)	0.158

*p-values from independent samples t-test

Values are mean (± standard deviation) of changes in percentage levels (%)

Table 4.12 Absolute concentration of maternal plasma cholesteryl ester (CE) fatty acids during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	Group	Time	Time × group
14:0	17.8 (±6.1)	18.0 (±6.6)	0.822	19.9 (±7.0)	18.6 (±6.8)	0.302	37.4 (±37.4)	51.4 (±57.1)	0.183	0.219	**<0.001**	0.157
16:0	268.3 (±55.6)	258.2 (±66.6)	0.364	321.9 (±62.0)	295.7 (±72.0)	**0.045**	557.1 (±511.1)	672.1 (±627.9)	0.353	0.449	**<0.001**	0.273
18:0	16.4 (±6.1)	16.4 (±6.8)	0.937	16.0 (±8.7)	14.4 (±7.3)	0.325	35.6 (±44.2)	49.5 (±61.0)	0.231	0.282	**<0.001**	0.190
20:0	3.0 (±1.7)	3.1 (±1.7)	0.724	11.8 (±11.5)	11.7 (±11.7)	0.956	1.1 (±1.3)	1.8 (±2.0)	0.060	0.743	**<0.001**	0.787
SFA	305.57 (±63.43)	295.65 (±74.82)	0.434	369.57 (±74.17)	340.42 (±85.43)	0.060	631.18 (±591.80)	774.85 (±742.96)	0.323	0.410	**<0.001**	0.251
16:1n-7	71.0 (±35.6)	70.1 (±39.0)	0.896	106.4 (±43.3)	88.5 (±48.6)	**0.046**	140.6 (±70.1)	144.7 (±90.9)	0.816	0.758	**<0.001**	0.204
18:1n-9	459.2 (±113.6)	452.7 (±130.9)	0.772	576.9 (±128.8)	537.3 (±153.2)	0.147	854.2 (±549.4)	966.5 (±730.5)	0.423	0.570	**<0.001**	0.354
18:1n-9 Trans	31.6 (±7.7)	30.1 (±6.7)	0.258	25.2 (±5.1)	23.8 (±6.2)	0.202	46.0 (±35.0)	48.3 (±35.8)	0.767	0.925	**<0.001**	0.623
20:1n-9	3.9 (±2.5)	3.4 (±2.1)	0.267	2.8 (±2.0)	2.6 (±2.0)	0.537	4.8 (±7.9)	6.6 (±8.9)	0.325	0.488	**0.004**	0.281
MUFA	565.76 (±146.57)	556.43 (±166.19)	0.744	711.20 (±161.65)	652.18 (±191.50)	0.085	1045.63 (±650.33)	1166.07 (±849.93)	0.462	0.655	**<0.001**	0.318
18:3n-3	18.4 (±7.7)	19.3 (±7.7)	0.496	21.3 (±9.5)	20.1 (±10.2)	0.537	32.1 (±16.7)	40.9 (±30.4)	0.104	0.159	**<0.001**	0.101
20:5n-3 (EPA)	11.8 (±6.6)	13.4 (±6.9)	0.207	11.8 (±7.0)	17.3 (±7.3)	**<0.001**	12.1 (±5.5)	21.8 (±10.5)	**<0.001**	**<0.001**	**<0.001**	**0.003**
22:5n-3 (DPA)	5.0 (±3.8)	4.1 (±2.8)	0.107	3.7 (±1.3)	3.4 (±1.8)	0.315	2.3 (±2.9)	4.0 (±4.7)	**0.042**	0.473	**0.010**	**0.040**
22:6n-3 (DHA)	15.0 (±4.6)	15.6 (±5.6)	0.560	15.9 (±4.4)	18.4 (±5.9)	**0.016**	20.3 (±9.4)	30.6 (±19.9)	**0.003**	**0.001**	**<0.001**	**0.017**
Total n-3	50.27 (17.57)	52.37 (16.90)	0.505	52.69 (17.70)	58.85 (18.49)	0.078	66.71 (29.81)	97.30 (58.26)	**0.003**	**0.002**	**<0.001**	**0.016**
18:2n-6	1097.7 (±254.0)	1086.9 (±274.8)	0.822	1318.6 (±324.0)	1238.5 (±315.6)	0.194	1439.1 (±302.8)	1375.3 (±473.9)	0.461	0.410	**<0.001**	0.226
18:3n-6	15.6 (±6.2)	13.8 (±7.5)	0.167	12.3 (±10.7)	10.4 (±9.7)	0.338	18.5 (±7.9)	16.0 (±6.5)	0.113	0.186	**<0.001**	0.501
20:2n-6	2.6 (±2.7)	1.9 (±1.4)	0.066	1.9 (±0.9)	1.7 (±1.2)	0.326	2.9 (±4.0)	6.9 (±23.5)	0.285	0.391	0.125	0.251
20:3n-6	22.7 (±5.9)	20.3 (±6.5)	**0.038**	24.5 (±6.5)	19.7 (±7.7)	**0.001**	27.2 (±7.3)	23.3 (±8.4)	**0.021**	**0.005**	**<0.001**	0.152
20:4n-6 (ARA)	150.0 (±40.3)	135.9 (±46.0)	0.076	163.1 (±40.7)	128.9 (±36.6)	**<0.001**	167.1 (±50.1)	145.7 (±47.5)	**0.044**	**0.025**	**0.001**	**0.002**
Total n-6	1288.64 (±286.88)	1258.89 (±315.18)	0.589	1520.43 (±355.07)	1399.21 (±342.53)	0.073	1654.79 (±328.36)	1567.20 (±521.71)	0.357	0.275	**<0.001**	0.136
PUFA	1338.91 (±297.73)	1311.26 (±325.54)	0.627	1573.11 (±366.73)	1458.06 (±353.67)	0.098	1721.50 (±344.90)	1664.50 (±561.95)	0.575	0.410	**<0.001**	0.188
Total fatty acids	2210.25 (±468.50)	2163.34 (±539.04)	0.611	2653.88 (±565.57)	2450.65 (±604.94)	0.073	3398.31 (±1439.15)	3605.42 (±1982.54)	0.581	0.925	**<0.001**	0. 304
ARA/DHA	10.33 (±2.21)	9.15 (±2.61)	**0.008**	10.48 (±1.92)	7.42 (±2.11)	**<0.001**	9.50 (±3.41)	5.89 (±2.44)	**<0.001**	**<0.001**	**<0.001**	**0.002**
ARA/EPA	15.04 (±6.77)	11.66 (±4.35)	**0.001**	15.97 (±5.76)	8.76 (±4.05)	**<0.001**	16.33 (±7.48)	7.60 (±3.04)	**<0.001**	**<0.001**	**0.013**	**0.001**
ARA/(DHA+EPA)	5.96 (±1.51)	4.99 (±1.47)	**<0.001**	6.17 (±1.37)	3.91 (±1.22)	**<0.001**	5.83 (±2.05)	3.21 (±1.20)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
n-6/n-3	27.25 (±6.92)	25.20 (±6.15)	0.088	30.41 (±7.69)	25.38 (±8.24)	**0.001**	28.21 (±9.30)	18.87 (±6.70)	**<0.001**	**<0.001**	**<0.001**	**0.012**

* *p*-values from independent samples t-test

Values are mean (± standard deviation) absolute concentrations (µg fatty acid/mL plasma) in CE

Table 4.13 Changes in cholesteryl ester (CE) fatty acid absolute concentration for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	p^*	Control (n = 41)	Salmon (n = 47)	p^*
20:5n-3 (EPA)	-0.13 (±7.34)	3.56 (±8.09)	**0.015**	1.5 (±4.4)	7.5 (±8.5)	**<0.001**
22:6n-3 (DHA)	1.00 (±4.85)	2.39 (±6.45)	0.209	6.04 (±9.18)	14.11 (±18.16)	**0.012**
20:4n-6 (ARA)	13.11 (±37.20)	-9.09 (±38.14)	**0.003**	23.06 (±47.18)	6.18 (±28.77)	**0.043**

*p-values from independent samples t-test

Values are mean (± standard deviation) of changes in absolute concentration (µg/mL)

4.3.1.4 Fatty acid composition of plasma non-esterified fatty acids (NEFA)

Table 4.14 describes the maternal plasma NEFA fatty acid composition expressed as percentages (%) of each fatty acid, for the two groups during pregnancy (20, 34, and 38 weeks gestation). Repeated measures ANOVA identified a significant effect of time on percentages of many fatty acids and a significant effect of group on the percentages of several fatty acids but particularly EPA, DPA, DHA, and total n-3 PUFA. There were significant time x group interactions for EPA, total n-3 PUFA, and ARA. At 20 weeks gestation the salmon group had a significantly higher percentages of DHA and total n-3 PUFA in plasma NEFA compared to the control group. At 34 and 38 weeks gestation the salmon group exhibited significantly higher percentages of EPA, DPA, DHA and total n-3 PUFA. The percentage of EPA decreased and that of DHA increased during pregnancy in the control group (p for trend = 0.042, < 0.001), whereas they both increased in the salmon group (p for trend = 0.018, < 0.001). The changes in % EPA in plasma NEFA were significantly different between groups (Table 4.15). Also, plasma NEFA % ARA increased significantly in both groups (p for trend < 0.001 for the control, and 0.014 for the salmon group), but the increase was significantly greater in the control group (Table 4.15).

Table 4.6 describes the absolute concentration (μg/mL plasma) of plasma NEFA fatty acids for both groups during pregnancy. Repeated measures ANOVA identified significant effects of time on the concentrations of all fatty acids detected in NEFA and in total plasma NEFA. There were significant effects of group on the concentrations of EPA, DHA, and total n-3 PUFA. There was a significant time x group interaction only for EPA. There were no significant differences in concentrations of individual NEFA at baseline. At 34 and 38 weeks gestation the salmon group had a higher absolute concentration of EPA, and at 38 weeks of DHA and total n-3 PUFA. The concentrations of EPA and DHA increased over time in both groups (all p for trend < 0.001 for both groups), but the increase was greater in the salmon group. The change in EPA and DHA concentrations in plasma NEFA from week 20 to week 38 were significantly different between groups (p = 0.001 and 0.048, respectively) (Table 4.17). Furthermore the increase in ARA from week 20 to week 38 was smaller in the salmon group (Table 4.17). Table 4.16 includes the ratios of fatty acids of interest in the plasma NEFA fraction. ARA/EPA, ARA/(EPA+DHA), and n-6 PUFA/n-3 PUFA were affected by time, ARA/EPA, ARA/DHA and n-6 PUFA/n-3 PUFA by group, and there was a significant time x group interaction for ARA/EPA.

Table 4.14 Maternal plasma non-esterified fatty acid (NEFA) content (%) during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	Group	Time	Time × group
14:0	1.23 (±0.40)	1.39 (±0.41)	**0.035**	1.30 (±0.32)	1.26 (±0.30)	0.439	1.44 (±0.37)	1.43 (±0.38)	0.906	0.542	**0.003**	0.059
16:0	24.12 (±3.38)	25.02 (±2.06)	0.078	26.34 (±1.82)	26.26 (±1.76)	0.822	25.82 (±1.84)	25.78 (±2.09)	0.928	0.171	**<0.001**	0.061
18:0	14.59 (±3.02)	15.73 (±2.88)	0.036	13.71 (±2.53)	14.93 (±3.60)	**0.043**	12.57 (±2.24)	13.56 (±2.91)	0.082	**0.043**	**<0.001**	0.967
20:0	0.98 (±0.47)	1.12 (±0.47)	0.110	0.40 (±0.35)	0.56 (±0.59)	0.082	1.49 (±0.34)	1.66 (±0.38)	**0.031**	**0.032**	**<0.001**	0.783
SFA	40.93 (±6.04)	43.26 (±4.02)	**0.013**	41.75 (±3.18)	43.00 (±4.11)	0.077	41.32 (±3.04)	42.42 (±3.84)	0.142	**0.018**	0.693	0.332
16:1n-7	2.74 (±0.95)	2.62 (±0.81)	0.451	2.93 (±0.82)	2.59 (±0.69)	0.019	3.21 (±0.95)	2.73 (±0.93)	**0.018**	0.087	**0.013**	0.064
18:1n-9	31.81 (±4.96)	31.08 (±4.05)	0.376	31.62 (±3.60)	29.62 (±4.56)	**0.012**	32.39 (±3.55)	30.27 (±4.21)	**0.013**	**0.035**	0.486	0.178
18:1n-9 Trans	3.08 (±3.21)	2.40 (±0.83)	0.108	2.18 (±0.34)	2.01 (±0.44)	**0.031**	2.41 (±0.48)	2.33 (±0.58)	0.483	**0.040**	**0.020**	0.102
20:1n-9	1.56 (±3.32)	0.99 (±0.82)	0.192	0.39 (±0.07)	0.38 (±0.10)	0.648	0.59 (±0.20)	0.75 (±0.44)	**0.036**	0.116	**0.003**	0.051
MUFA	39.19 (±6.40)	37.09 (±4.63)	**0.040**	37.11 (±4.33)	43.60 (±5.32)	**0.008**	38.60 (±4.27)	36.08 (±5.02)	**0.013**	**0.006**	**0.003**	0.980
20:5n-3 (EPA)	0.29 (±0.20)	0.33 (±0.17)	0.188	0.22 (±0.10)	0.41 (±0.19)	**<0.001**	0.22 (±0.09)	0.44 (±0.27)	**<0.001**	**<0.001**	0.802	**0.002**
22:5n-3 (DPA)	0.38 (±0.22)	0.41 (±0.17)	0.425	0.43 (±0.14)	0.54 (±0.19)	**0.001**	0.42 (±0.13)	0.54 (±0.18)	**0.001**	**0.001**	**<0.001**	0.269
22:6n-3 (DHA)	1.89 (±1.28)	2.45 (±1.34)	**0.021**	3.08 (±0.90)	4.12 (±1.21)	**<0.001**	2.85 (±0.85)	4.29 (±1.42)	**<0.001**	**<0.001**	**<0.001**	0.113
Total n-3	2.83 (±1.26)	3.40 (±1.25)	**0.014**	4.64 (±1.14)	6.01 (±1.50)	**<0.001**	3.48 (±1.02)	5.26 (±1.81)	**<0.001**	**<0.001**	**<0.001**	**0.033**
18:2n-6	11.97 (±2.24)	12.58 (±1.81)	0.102	12.72 (±2.07)	12.68 (±1.59)	0.920	12.71 (±1.74)	12.43 (±1.35)	0.400	0.784	0.051	**0.005**
18:3n-6	0.57 (±0.76)	0.48 (±0.49)	0.425	0.06 (±0.22)	0.19 (±1.23)	0.411	0.11 (±0.05)	0.08 (±0.08)	0.083	0.690	**<0.001**	0.102
20:3n-6	0.43 (±0.17)	0.45 (±0.17)	0.612	0.48 (±0.12)	0.47 (±0.18)	0.703	0.48 (±0.18)	0.45 (±0.13)	0.519	0.705	0.140	0.147
20:4n-6 (ARA)	2.52 (±0.94)	2.70 (±1.03)	0.315	3.22 (±0.89)	3.01 (±0.85)	0.210	3.31 (±0.95)	3.27 (±0.88)	0.849	0.620	**<0.001**	**0.004**
Total n-6	17.05 (±7.26)	16.25 (±2.10)	0.410	16.49 (±2.12)	16.39 (±2.13)	0.802	16.60 (±2.03)	16.24 (±1.34)	0.321	0.260	0.430	0.623
PUFA	19.88 (±7.18)	19.65 (±2.72)	0.817	21.14 (±2.75)	22.40 (±2.74)	**0.017**	20.08 (±2.56)	21.50 (±2.59)	**0.012**	0.178	**0.023**	0.331

**p*-Values from independent samples t-test

Values are mean (± standard deviation) percentage levels (%) of total fatty acids in NEFA

Table 4.15 Changes of percentage non-esterified fatty acid levels (%NEFA) for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
20:5n-3 (EPA)	-0.07 (±0.21)	0.07 (±0.20)	**<0.001**	-0.06 (±0.20)	0.09 (±0.25)	**0.004**
22:6n-3 (DHA)	1.17 (±1.27)	1.61 (±1.46)	0.101	1.07 (±1.25)	1.72 (±1.95)	0.072
20:4n-6 (ARA)	0.70 (±1.13)	0.22 (±0.93)	**0.018**	0.96 (±1.02)	0.43 (±1.13)	**0.025**

**p*-values from independent samples t-test

Values are mean (± standard deviation) of changes in percentage levels (%)

Table 4.16 Absolute concentration of maternal plasma non-esterified fatty acids (NEFA) during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	**Group**	**Time**	**Time × group**
14:0	0.53 (±0.57)	0.65 (±0.69)	0.296	0.97 (±0.71)	0.82 (±0.63)	0.260	2.03 (±0.98)	1.80 (±0.94)	0.258	0.331	**<0.001**	0.226
16:0	10.52 (±10.35)	11.82 (±11.87)	0.525	18.91 (±10.68)	17.19 (±12.95)	0.451	35.39 (±11.81)	31.82 (±11.93)	0.162	0.269	**<0.001**	0.307
18:0	6.61 (±7.53)	7.58 (±7.98)	0.493	9.55 (±5.04)	9.70 (±9.86)	0.923	16.60 (±3.62)	15.72 (±3.64)	0.259	0.505	**<0.001**	0.501
20:0	0.43 (±0.45)	0.53 (±0.67)	0.326	0.29 (±0.39)	0.39 (±0.61)	0.292	2.01 (±0.71)	2.03 (±0.89)	0.915	0.503	**<0.001**	0.943
SFA	18.10 (±18.64)	20.58 (±20.94)	0.492	29.72 (±16.27)	28.11 (±22.99)	0.672	56.03 (±16.28)	51.36 (±16.74)	0.189	0.974	**<0.001**	0.350
16:1n-7	1.22 (±1.23)	1.29 (±1.51)	0.761	2.20 (±1.78)	1.70 (±1.29)	0.097	4.54 (±2.34)	3.60 (±2.23)	0.055	0.078	**<0.001**	0.098
18:1n-9	13.89 (±13.17)	14.99 (±15.58)	0.678	22.83 (±13.61)	19.37 (±13.95)	0.191	44.85 (±16.65)	38.85 (±18.47)	0.114	0.138	**<0.001**	0.239
18:1n-9 Trans	1.31 (±1.55)	1.15 (±1.24)	0.518	1.56 (±0.95)	1.33 (±1.00)	0.213	3.32 (±1.29)	2.99 (±1.65)	0.303	0.084	**<0.001**	0.992
20:1n-9	0.65 (±1.23)	0.46 (±0.50)	0.270	0.27 (±0.14)	0.27 (±0.26)	0.964	0.81 (±0.37)	0.92 (±0.74)	0.399	0.256	**<0.001**	0.068
MUFA	17.07 (±15.80)	17.89 (±18.64)	0.795	26.86 (±16.30)	22.67 (±16.33)	0.181	53.53 (±20.07)	46.36 (±22.33)	0.118	0.587	**<0.001**	0.399
20:5n-3 (EPA)	0.12 (±0.12)	0.15 (±0.12)	0.186	0.15 (±0.10)	0.24 (±0.19)	**0.003**	0.28 (±0.11)	0.48 (±0.23)	**<0.001**	**<0.001**	**<0.001**	**0.002**
22:5n-3 (DPA)	0.16 (±0.19)	0.19 (±0.21)	0.476	0.30 (±0.16)	0.34 (±0.30)	0.324	0.55 (±0.17)	0.61 (±0.18)	0.108	0.104	**<0.001**	0.686
22:6n-3 (DHA)	0.86 (±1.16)	1.14 (±1.31)	0.222	2.08 (±1.08)	2.67 (±2.15)	0.072	3.69 (±0.98)	4.88 (±1.45)	**<0.001**	**<0.001**	**<0.001**	0.094
Total n-3	1.25 (±1.40)	1.58 (±1.65)	0.240	3.18 (±1.64)	3.85 (±2.93)	0.142	4.52 (±1.17)	5.97 (±1.77)	**<0.001**	**<0.001**	**<0.001**	0.124
18:2n-6	5.10 (±4.55)	5.91 (±6.06)	0.410	9.18 (±5.64)	8.38 (±6.15)	0.479	17.46 (±6.24)	15.53 (±6.56)	0.164	0.284	**<0.001**	0.174
18:3n-6	0.26 (±0.37)	0.25 (±0.34)	0.906	0.05 (±0.20)	0.08 (±0.45)	0.582	0.14 (±0.09)	0.11 (±0.11)	0.109	0.623	**<0.001**	0.253
20:3n-6	0.17 (±0.13)	0.19 (±0.16)	0.473	0.34 (±0.19)	0.32 (±0.37)	0.746	0.63 (±0.24)	0.54 (±0.20)	0.061	0.063	**<0.001**	0.118
20:4n-6 (ARA)	1.05 (±1.02)	1.16 (±1.04)	0.562	2.17 (±1.10)	1.99 (±1.99)	0.562	4.27 (±0.97)	3.79 (±1.11)	**0.034**	0.135	**<0.001**	0.077
Total n-6	6.59 (±5.84)	7.52 (±7.43)	0.451	11.75 (±6.73)	10.80 (±8.19)	0.507	22.50 (±6.76)	19.97 (±7.40)	0.099	0.211	**<0.001**	0.162
PUFA	7.85 (±7.14)	9.10 (±8.99)	0.399	14.93 (±8.24)	14.65 (±10.96)	0.879	27.02 (±7.39)	25.94 (±8.44)	0.527	0.796	**<0.001**	0.408
Total fatty acids	43.01 (±40.82)	47.57 (±48.11)	0.576	71.51 (±39.93)	65.43 (±48.31)	0.473	136.58 (±41.98)	123.66 (±46.35)	0.176	0.251	**<0.001**	0.350
ARA/DHA	1.71 (±3.55)	1.10 (±0.76)	0.213	1.07 (±0.15)	0.75 (±0.17)	**<0.001**	1.18 (±0.19)	0.79 (±0.17)	**<0.001**	**0.041**	0.133	0.573
ARA/EPA	7.32 (±2.44)	7.31 (±2.76)	0.988	14.76 (±6.02)	8.11 (±4.59)	**<0.001**	17.07 (±6.99)	9.13 (±4.36)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ARA/(DHA+EPA)	1.90 (±2.20)	1.59 (±2.01)	0.430	1.00 (±0.16)	0.69 (±0.18)	**<0.001**	1.10 (±0.18)	0.72 (±0.16)	**<0.001**	0.164	**0.001**	0.384
n-6/n-3	6.75 (±3.45)	5.49 (±2.46)	**0.022**	3.71 (±0.84)	2.89 (±0.86)	**<0.001**	5.15 (±1.55)	3.44 (±1.26)	**<0.001**	**<0.001**	**<0.001**	0.326

p-values from independent samples t-test

Values are mean (± standard deviation) absolute concentrations (µg fatty acid/mL plasma) in NEFA

Table 4.17 Changes in non-esterified fatty acid (NEFA) absolute concentration for selected fatty acids of interest

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *
20:5n-3 (EPA)	0.03 (±0.16)	0.09 (±0.24)	0.113	0.17 (±0.15)	0.33 (±0.28)	**0.001**
22:6n-3 (DHA)	1.28 (±1.58)	1.56 (±2.82)	0.524	2.88 (±1.44)	3.69 (±2.19)	**0.048**
20:4n-6 (ARA)	1.18 (±1.55)	0.87 (±2.45)	0.436	3.29 (±1.41)	2.59 (±1.67)	**0.041**

*p-values from independent samples t-test

Values are mean (± standard deviation) of changes in absolute concentration (µg/mL)

4.3.1.5 Total plasma lipids and total plasma ARA, EPA, DPA and DHA

Figure 4.14 describes the total plasma PC, TAG, CE and NEFA concentrations (μg/mL) across pregnancy in the control group (values can be seen on Table 4.4, Table 4.8, Table 4.12, and Table 4.16). As it can be seen from the graph, higher absolute concentrations were observed for PC and CE, and then TAG. NEFA concentration was the lowest and on the graph is represented with a different scale from the other fractions. It was shown that total fatty acid concentrations in each lipid fraction changed significantly during pregnancy (all *p* for trend < 0.001). Total PC concentration increased (by 40%) from week 20 to week 34 gestation and then it decreased at 38 weeks (by 25% compared to 20 weeks). Total plasma TAG concentration increased from 20 to 34 weeks (by 70%) and remained stable thereafter. Interestingly, total plasma CE concentration increased progressively throughout pregnancy (by 54% at 38 weeks compared to 20 weeks), resulting in higher concentrations than any other fraction at 38 weeks. Total plasma NEFA concentration increased remarkably during pregnancy (by 220% at 38 weeks compared to 20 weeks). These data show that pregnancy is associated with increase of total blood lipid concentrations and that significant changes occur between mid to late pregnancy.

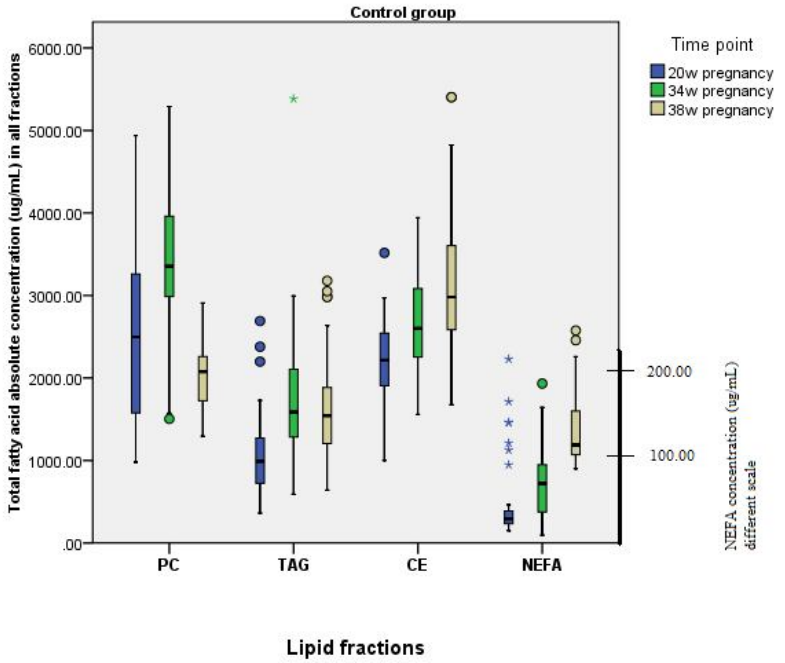


Figure 4.14 Total plasma PC, TAG, CE, and NEFA concentrations (μg/mL) across pregnancy in the control group

Note: NEFA is presented on a different scale; All *p* for trend < 0.001 (for each lipid class)

Table 4.18 describes the maternal total plasma EPA, DHA, DPA and ARA concentration during pregnancy calculated by summing the concentrations of each of these fatty acids of interest across the four plasma lipid fractions (i.e. TAG + PC + CE + NEFA). Repeated measures ANOVA showed significant effects of time on the total plasma concentration of all four fatty acids and significant group effects on the concentrations of EPA, DHA and DPA. There were significant time x group interactions for EPA and ARA. These were further explored by pair-wise comparisons between groups at each time point. There were no significant differences between the two groups at 20 weeks gestation. At 34 and 38 weeks gestation, the salmon group had lower total plasma ARA concentration compared to the control group. At 34 and 38 weeks gestation, the salmon group had higher total plasma EPA and DHA concentrations. Total plasma DPA concentration was significantly higher in the salmon group at 38 weeks. Total plasma EPA decreased over time during pregnancy in the control group (p for trend = 0.030), whereas it increased in the salmon group (p for trend = 0.001). Total plasma ARA decreased during pregnancy in both groups (both p for trend < 0.001), but the decrease was greater in the salmon group. Total plasma DHA increased and then decreased during pregnancy in both groups (both p for trend < 0.001). Total plasma DPA decreased during pregnancy in the control (p for trend < 0.001).

Table 4.18 Maternal total plasma concentrations (μg/mL) of fatty acids of interest during pregnancy

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 61)	Salmon (n = 62)	*p* *	Control (n = 55)	Salmon (n = 56)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	**Group**	**Time**	**Time × group**
Total EPA	30.4 (±19.9)	34.6 (±21.0)	0.265	30.3 (±14.9)	50.3 (±24.2)	**<0.001**	23.1 (±9.9)	45.6 (±22.6)	**<0.001**	**<0.001**	**0.001**	**0.003**
Total DHA	129.3 (±55.7)	141.5 (±63.9)	0.273	163 (±41.9)	205.9 (±71.9)	**<0.001**	110.9 (±31.3)	165.9 (±68.1)	**<0.001**	**<0.001**	**<0.001**	0.201
Total DPA	25.1 (±12.4)	24.4 (±11.2)	0.728	26.8 (±8.5)	29.8 (±9.8)	0.083	16.6 (±7.9)	22.2 (±11.9)	**0.013**	**0.029**	**<0.001**	0.l74
Total ARA	412.1 (±141.7)	376.5 (±144.3)	0.180	477.6 (±111.5)	390.3 (±109.1)	**<0.001**	359.8 (±84.6)	320.8 (±94.0)	**0.048**	0.080	**<0.001**	**0.014**

p-values from independent samples t-test

Values are mean (± standard deviation) absolute concentrations (μg fatty acid/mL plasma) of each fatty acid concentration in all lipid fractions (PC+TAG+CE+NEFA)

4.3.2 Fatty acid composition of maternal peripheral blood mononuclear cells (PBMC) during pregnancy

Table 4.19 describes the maternal PBMC fatty acid composition expressed as percentages (%) of each fatty acid, for the two groups during pregnancy (20, 34, and 38 weeks gestation). Repeated measures ANOVA identified a significant effect of time on percentages of many fatty acids and a significant effect of group on the percentages of EPA, DHA, total n-3 PUFA, linoleic acid (18:2n-6) and ARA. There were significant time x group interactions for oleic acid (18:1n-9), EPA, DPA, DHA, total n-3 PUFA, and totals n-6 PUFA. At 20 weeks gestation the salmon group had a significantly higher percentage of linoleic acid in PBMC lipids compared to the control group. At 34 and 38 weeks gestation the salmon group exhibited significantly higher percentages of EPA, DPA, DHA and total n-3 PUFA in PBMC. The percentages of EPA, DPA and DHA decreased during pregnancy in the control group (all p for trend ≤ 0.001), while these decreases were prevented in the salmon group for EPA and DPA (p for trend = 0.598, 0.051), whereas percentage DHA increased in the salmon group (p for trend = 0.005) (Figure 4.15). The changes in % EPA, DPA and DHA in PBMC were significantly different between groups (Table 4.20). Also, PBMC % ARA decreased significantly in both groups (p for trend = 0.005 for control, and < 0.001 for salmon group), and the decrease tended to be greater in the salmon group but not significantly so (Table 4.20).

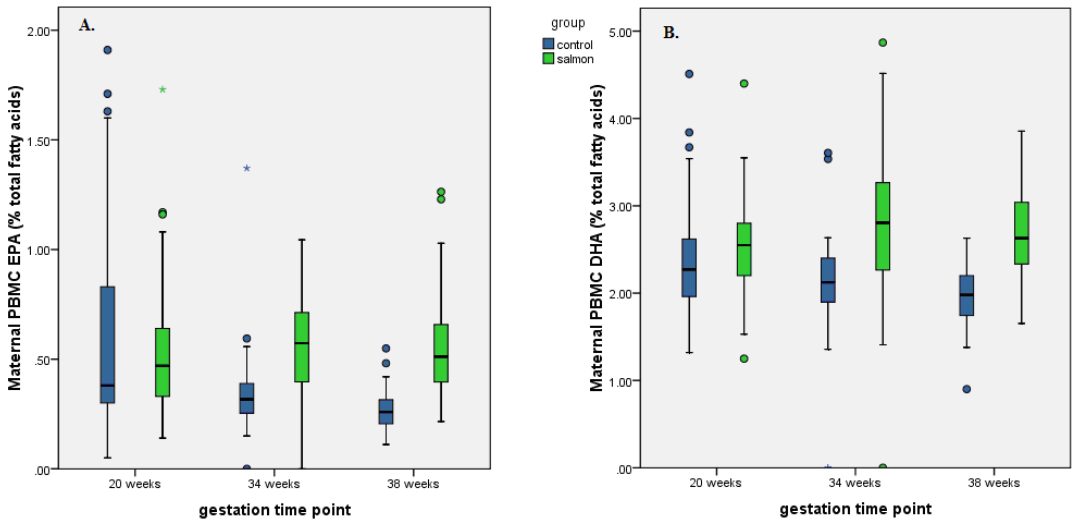


Figure 4.15 Maternal PBMC EPA (A) and DHA (B) percentages during pregnancy for both groups
$p < 0.05$; for exact values see text and Table 4.19

Figure 4.16 shows the correlation between maternal plasma PC and PBMC fatty acid composition (both expressed as percentages of total fatty acids). Data from all time points during pregnancy in both groups showed that maternal plasma PC EPA, DHA, DPA, and ARA percentage levels correlated significantly with maternal PBMC EPA, DHA, DPA and ARA percentage levels (Figure 4.16, all $p < 0.001$). Data from individual time points in both groups during pregnancy also showed significant correlations. Figure 4.17 presents data from 38 weeks gestation in both groups, showing significant correlations between maternal plasma PC and PBMC content for EPA and DHA (all $p < 0.001$). These data show that plasma PC can be used to indicate the fatty acid composition in cells.

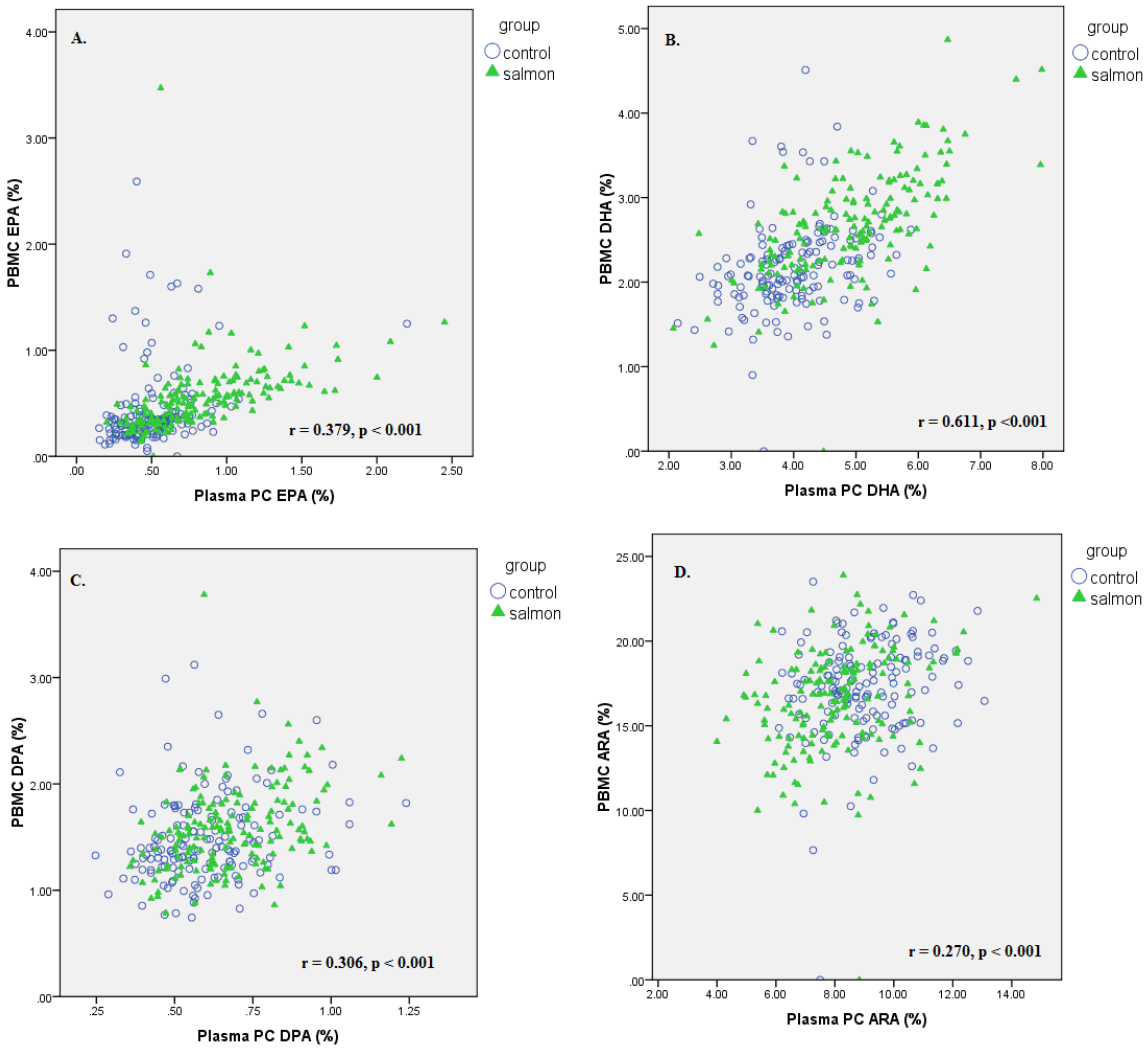


Figure 4.16 Correlation between maternal plasma PC (%) and PBMC content of EPA (A), DHA (B), DPA (C), ARA (D) for all time points in both groups

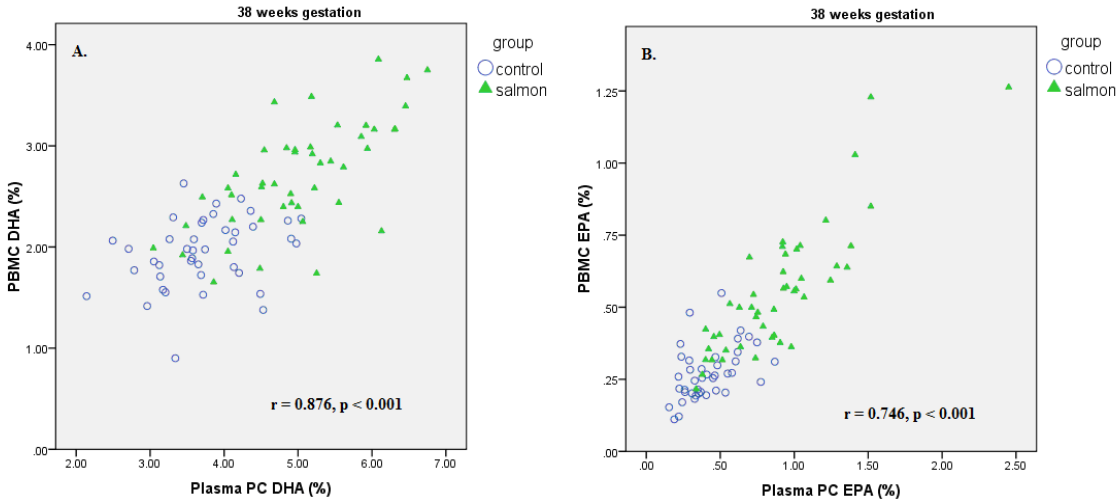


Figure 4.17 Correlation between maternal plasma PC (%) and PBMC content of EPA (A), DHA (B) at 38 weeks gestation in both groups

Table 4.19 Maternal peripheral blood mononuclear cell (PBMC) fatty acid composition (%) during pregnancy- between group comparisons

Fatty acids	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control (n = 57)	Salmon (n = 61)	*p* *	Control (n = 53)	Salmon (n = 55)	*p* *	Control (n = 41)	Salmon (n = 47)	*p* *	Group	Time	Time × group
14:0	1.05 (±0.69)	0.94 (±0.77)	0.399	1.29 (±0.73)	1.31 (±0.84)	0.883	0.98 (±0.89)	1.20 (±1.33)	0.371	0.682	0.082	0.371
16:0	20.78 (±2.30)	20.15 (±3.30)	0.233	21.89 (±1.71)	21.97 (±1.32)	0.793	23.23 (±2.41)	23.01 (±2.04)	0.634	0.800	**<0.001**	0.462
18:0	21.61 (±5.03)	21.27 (±4.73)	0.707	19.46 (±3.66)	19.97 (±4.00)	0.494	19.04 (±2.86)	18.47 (±2.51)	0.329	0.402	**<0.001**	0.430
20:0	0.58 (±0.44)	0.57 (±0.39)	0.933	0.68 (±0.22)	0.70 (±0.26)	0.638	0.46 (±0.16)	0.51 (±0.20)	0.270	0.928	**0.001**	0.684
SFA	44.02 (±5.43)	42.93 (±5.18)	0.262	43.32 (±3.47)	43.94 (±4.65)	0.429	43.71 (±3.85)	43.18 (±3.50)	0.505	0.458	0.946	0.095
16:1n-7	0.85 (±0.56)	0.76 (±0.38)	0.311	0.92 (±0.41)	0.79 (±0.28)	0.044	0.93 (±0.45)	0.92 (±0.48)	0.971	0.606	0.083	0.783
18:1n-9	17.30 (±2.64)	17.74 (±2.39)	0.341	18.82 (±1.96)	18.29 (±1.81)	0.150	20.17 (±2.08)	19.61 (±2.00)	0.200	0.894	**<0.001**	**0.016**
18:1n-9 Trans	1.90 (±1.13)	1.64 (±0.64)	0.132	2.13 (±0.40)	1.88 (±0.31)	**0.001**	1.58 (±0.26)	1.48 (±0.23)	0.066	**0.007**	**<0.001**	0.411
20:1n-9	0.75 (±0.30)	0.71 (±0.31)	0.532	1.38 (±0.59)	1.21 (±0.47)	0.099	71 (±0.18)	0.70 (±0.27)	0.898	0.108	**<0.001**	0.241
MUFA	20.79 (±2.74)	20.85 (±2.84)	0.901	23.25 (±1.96)	22.17 (±1.95)	**0.005**	23.38 (±2.45)	22.71 (±2.32)	0.191	0.367	**<0.001**	**0.040**
20:5n-3 (EPA)	0.63 (±0.53)	0.58 (±0.48)	0.563	0.34 (±0.19)	0.55 (±0.21)	**<0.001**	0.27 (±0.09)	0.55 (±0.22)	**<0.001**	**0.001**	**0.001**	**0.017**
22:5n-3 (DPA)	1.70 (±0.48)	1.67 (±0.48)	0.742	1.36 (±0.30)	1.56 (±0.37)	**0.003**	1.31 (±0.29)	1.48 (±0.29)	**0.007**	0.084	**<0.001**	**0.034**
22:6n-3 (DHA)	2.38 (±0.60)	2.55 (±0.52)	0.118	2.13 (±0.52)	2.79 (±0.81)	**<0.001**	1.95 (±0.35)	2.70 (±0.54)	**<0.001**	**<0.001**	**0.002**	**<0.001**
Total n-3	4.72 (±1.36)	4.80 (v1.13)	0.731	3.83 (±0.86)	4.91 (v1.25)	**<0.001**	3.53 (±0.53)	4.73 (±0.88)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
18:2n-6	9.71 (±3.29)	10.96 (±3.42)	**0.045**	10.43 (±2.50)	10.86 (±2.62)	0.376	10.17 (±2.45)	10.94 (±2.10)	0.115	**0.013**	0.776	0.148
20:2n-6	0.60 (±0.37)	0.51 (±0.22)	0.114	0.49 (±0.17)	0.50 (±0.16)	0.734	0.52 (±0.27)	0.52 (±0.34)	0.999	0.077	0.105	0.107
20:3n-6	2.03 (±0.58)	2.01 (±0.45)	0.870	1.72 (±0.33)	1.62 (±0.33)	0.136	2.12 (±1.19)	2.20 (±1.67)	0.790	0.639	**0.003**	0.615
20:4n-6 (ARA)	18.13 (±2.52)	17.93 (±3.00)	0.695	16.71 (±3.14)	15.64 (±3.29)	0.089	16.58 (±2.93)	15.71 (±2.25)	0.121	**0.041**	**<0.001**	0.589
Total n-6	30.47 (±3.76)	31.42 (±3.45)	0.156	29.34 (±3.38)	28.63 (±3.90)	0.317	29.38 (±2.69)	29.37 (±2.57)	0.983	0.942	**<0.001**	**0.027**
PUFA	35.19 (±4.05)	36.22 (±3.46)	0.140	33.17 (±3.83)	33.54 (±4.56)	0.651	32.91 (±2.91)	34.11 (±2.97)	0.061	0.173	**<0.001**	0.220
ARA/DHA	7.94 (±1.73)	7.35 (±1.90)	0.080	8.07 (±1.55)	5.93 (±1.72)	**<0.001**	8.65 (±1.60)	6.00 (±1.16)	**<0.001**	**<0.001**	**0.008**	**<0.001**
ARA/EPA	53.16 (±52.62)	43.45 (±22.33)	0.190	57.22 (±21.52)	32.69 (±14.80)	**<0.001**	69.88 (±30.61)	32.34 (±12.39)	**<0.001**	**<0.001**	0.214	**0.035**
ARA/(DHA+EPA)	6.50 (±1.69)	6.11 (±1.72)	0.210	6.98 (±1.32)	4.98 (±1.50)	**<0.001**	7.57 (±1.51)	5.00 (±1.03)	**<0.001**	**<0.001**	0.070	**<0.001**
n-6/n-3	6.91 (±1.80)	6.84 (±1.48)	0.821	7.92 (±1.35)	6.17 (±1.57)	**<0.001**	8.48 (±1.22)	6.40 (±1.24)	**<0.001**	**<0.001**	**0.001**	**<0.001**

*p-values from independent samples t-test

Values are mean (± standard deviation) percentage levels (%) of total fatty acids in PBMC

Table 4.20 Peripheral blood mononuclear cell (PBMC) fatty acid changes

Fatty Acids	34-20 week change			38-20 week change		
	Control (n = 53)	Salmon (n = 55)	p^*	Control (n = 41)	Salmon (n = 47)	p^*
20:5n-3 (EPA)	-0.31 (±0.57)	-0.02 (±0.49)	**0.006**	-0.37 (±0.59)	-0.06 (±0.55)	**0.012**
22:6n-3 (DHA)	-0.25 (±0.75)	0.27 (±0.68)	**<0.001**	-0.41 (±0.55)	0.07 (±0.62)	**<0.001**
20:4n-6 (ARA)	-1.07 (±3.01)	-2.05 (±3.25)	0.113	-1.61 (±3.36)	-1.89 (±3.01)	0.680
22:5n-3 (DPA)	-0.34 (±0.56)	-0.07 (±0.51)	**0.014**	-0.40 (±0.56)	-0.17 (±0.51)	**0.048**

*p-Values from independent samples t-test

Values are mean (± standard deviation) of changes in percentage levels (%)

4.3.3 Fatty acid composition of placenta and umbilical cord

Table 4.21 shows the fatty acid composition of placenta and umbilical cord tissues in both groups, expressed as percentage (%) of each fatty acid. The salmon group had significantly higher percentages of α-linolenic acid (18:3n-3), EPA, DPA, DHA, DPA, and total n-3 PUFA compared to the control group and significantly lower percentages of ARA and total n-6 PUFA. Finally, the fatty acid ratios calculated were all significantly lower in the salmon group compared to the control group (all $p < 0.001$).

The percentages of DPA, DHA and total n-3 PUFA were significantly higher in umbilical cord tissue of the salmon group compared to the control group. The percentage of ARA in cord tissue lipids did not differ between the groups. All fatty acid ratios calculated were significantly lower in the salmon group compared to control group (all $p < 0.001$, apart from ARA/EPA for which $p = 0.022$).

Table 4.21 Placenta and cord tissue fatty acid composition expressed as percentage levels (%)

Fatty Acids	Placenta tissue			Cord tissue		
	Control (n = 45)	Salmon (n = 47)	p^*	Control (n = 41)	Salmon (n = 45)	p^*
14:0	0.42 (±0.09)	0.45 (±0.15)	0.286	1.45 (±0.34)	1.45 (±0.46)	0.964
16:0 (%)	25.25 (±0.96)	25.40 (±0.89)	0.458	26.88 (±1.98)	27.47 (±2.59)	0.247
18:0 (%)	12.21 (±0.77)	12.06 (±0.96)	0.416	16.55 (±1.60)	16.28 (±1.78)	0.465
20:0 (%)	0.11 (±0.02)	0.11 (±0.03)	0.315	0.40 (±0.17)	0.43 (±0.19)	0.505
24:0 (%)	-	-		4.45 (±0.88)	4.34 (±0.75)	0.565
SFA	38.00 (±0.67)	38.03 (±0.90)	0.868	49.73 (±1.97)	49.98 (±2.30)	0.600
16:1n-7 (%)	0.84 (±0.20)	0.83 (±0.19)	0.701	1.90 (±0.36)	1.84 (±0.59)	0.574
18:1n-9 (%)	11.14 (±0.94)	11.19 (±0.94)	0.787	18.26 (±2.03)	17.14 (±1.94)	**0.011**
18:1n-9 Trans (%)	1.78 (±0.19)	1.70 (±0.15)	0.026	3.89 (±0.68)	3.56 (±0.51)	**0.013**
20:1n-9 (%)	0.23 (±0.03)	0.24 (±0.04)	0.228	0.95 (±0.32)	0.86 (±0.22)	0.128
MUFA	13.99 (±1.16)	13.96 (±1.06)	0.885	25.00 (±2.92)	23.40 (±2.75)	**0.011**
18:3n-3	0.11 (±0.03)	0.13 (±0.04)	**0.009**	-	-	
20:5n-3 (EPA) (%)	0.18 (±0.05)	0.33 (±0.13)	**<0.001**	0.31 (±0.26)	0.43 (±0.56)	0.192
22:5n-3 (DPA) (%)	0.95 (±0.17)	1.11 (±0.20)	**<0.001**	0.57 (±0.24)	0.71 (±0.29)	**0.017**
22:6n-3 (DHA)	4.92 (±0.79)	5.93 (±0.94)	**<0.001**	3.65 (±0.86)	4.30 (±0.88)	**0.001**
Total n-3	6.15 (0.77)	7.50 (0.97)	**<0.001**	4.53 (1.07)	5.44 (1.22)	**<0.001**
18:2n-6 (%)	11.22 (±1.43)	11.75 (±1.59)	0.095	4.48 (±1.38)	5.06 (±1.54)	0.073
18:3n-6	0.13 (±0.03)	0.13 (±0.03)	0.298	0.21 (±0.11)	0.26 (±0.36)	0.383
20:2n-6 (%)	0.42 (±0.05)	0.44 (±0.06)	**0.033**	0.41 (±0.11)	0.52 (±0.26)	**0.010**
20:3n-6 (%)	6.19 (±0.91)	5.88 (±0.96)	0.114	2.41 (±0.43)	2.57 (±0.49)	0.116
20:4n-6 (ARA) (%)	23.89 (±1.38)	22.32 (±2.11)	**<0.001**	12.07 (±2.30)	11.74 (±1.72)	0.450
Total n-6	41.85 (±1.32)	40.51 (±1.50)	**<0.001**	19.58 (±3.13)	20.15 (±2.76)	0.372
22:2n-9 (%)	-	-		1.16 (±0.36)	1.03 (±0.32)	0.071
PUFA	48.01 (±1.34)	48.01 (±1.59)	0.985	25.27 (±3.79)	26.62 (±3.45)	0.088
ARA/DHA	4.97 (±0.81)	3.85 (±0.68)	**<0.001**	3.38 (±0.46)	2.79 (±0.41)	**<0.001**
ARA/EPA	148.08 (56.23)	80.94 (±38.73)	**<0.001**	54.04 (±29.86)	40.72 (±23.08)	**0.022**
ARA/(DHA+EPA)	4.79 (±0.76)	3.64 (±0.64)	**<0.001**	3.12 (±0.48)	2.55 (±0.44)	**<0.001**
n-6/n-3	6.92 (±1.00)	5.50 (±0.81)	**<0.001**	4.46 (±0.76)	3.82 (±0.68)	**<0.001**

*p-values from independent samples t-test

Values are mean (± standard deviation) percentage levels (%) of total fatty acids in placenta and cord tissues

4.3.4 Summary of the data

Table 4.22 summarises the findings of this study with regard to lipid concentrations and the fatty acids of principal interest (ARA, EPA, DPA, DHA). Pregnancy resulted in increased total plasma fatty acid concentrations in all lipid fractions (especially for CE and NEFA) apart from the PC fraction for which total plasma fatty acids increased initially (between 20 to 34 weeks) and then decreased (at 38 weeks). These data show that the pregnancy associated hyperlipidemia occurred and that it was not affected by the salmon intervention. Total plasma ARA, EPA, DPA and DHA concentrations decreased during pregnancy. The salmon intervention reversed the change in LC n-3 PUFA, resulting in increased total plasma EPA, DPA and DHA concentrations. However, for ARA the salmon intervention resulted in a further decrease in total plasma concentration. ARA decreased in plasma PC, TAG (%), and PBMC, whereas it increased in plasma CE (μg/mL) and NEFA during pregnancy. The salmon intervention resulted in further decrease of ARA in plasma PC (%) and CE, and in PBMC. Also, salmon decreased ARA in placental tissue. Pregnancy resulted in decreased EPA in plasma PC, TAG (%) and CE (%), and PBMC, whereas NEFA EPA (μg/mL) increased during pregnancy. The salmon intervention resulted in increased percentage levels and concentrations of EPA in all plasma lipid fractions, and in PBMC and placenta tissue. Pregnancy resulted in decreased DPA in maternal plasma PC and CE, and PBMC, whereas it resulted in increased DPA in plasma NEFA and TAG (μg/mL). The effect of the salmon intervention was to increase DPA in all plasma lipid fractions and PBMC during pregnancy, and in placenta and cord tissues at delivery. Pregnancy decreased DHA in plasma PC and in PBMC, whereas it increased DHA in plasma TAG (μg/mL), CE (μg/mL), and NEFA. The effect of the salmon intervention on DHA was to reverse the pregnancy-associated decrease in plasma PC and PMBC, resulting in increased levels, and it further increased DHA in plasma TAG, CE, and NEFA. The salmon intervention also resulted in higher DHA status in cord and placenta tissue at delivery.

Table 4.22 The effect of pregnancy and the effect of the salmon intervention on maternal total lipid concentrations and the fatty acids of principal interest

	Effect of pregnancy	Effect of salmon
Total plasma PC (μg/mL)	↑ then ↓	↔
Total plasma TAG (μg/mL)	↑	↔
Total plasma CE (μg/mL)	↑	↔
Total plasma NEFA (μg/mL)	↑	↔
Total plasma ARA (μg/mL)	↓	↓
Total plasma EPA (μg/mL)	↓	↑
Total plasma DPA (μg/mL)	↓	↑
Total plasma DHA (μg/mL)	↑ then ↓	↑
ARA in:		
Plasma PC (μg/mL)	↑ then ↓	↔
Plasma PC (%)	↓	↓
Plasma TAG (μg/mL)	↔	↔
Plasma TAG (%)	↓	↔
Plasma CE (μg/mL)	↑	↓
Plasma CE (%)	↓	↓
Plasma NEFA (μg/mL)	↑	↔
Plasma NEFA (%)	↑	↔
PBMC	↓	↓
Placenta	-	↓
Cord tissue	-	↔
EPA in:		
Plasma PC (μg/mL)	↓	↑
Plasma PC (%)	↓	↑
Plasma TAG (μg/mL)	↔	↑
Plasma TAG (%)	↓	↑
Plasma CE (μg/mL)	↔	↑
Plasma CE (%)	↓	↑
Plasma NEFA (μg/mL)	↑	↑
Plasma NEFA (%)	↔	↑
PBMC	↓	↑
Placenta	-	↑
Cord tissue	-	↔
DPA in:		
Plasma PC (μg/mL)	↓	↑
Plasma PC (%)	↓	↔
Plasma TAG (μg/mL)	↑	↑
Plasma TAG (%)	↔	↑
Plasma CE (μg/mL)	↓	↑
Plasma CE (%)	↓	↔
Plasma NEFA (μg/mL)	↑	↔
Plasma NEFA (%)	↑	↑
PBMC	↓	↑
Placenta	-	↑
Cord tissue	-	↑
DHA in:		
Plasma PC (μg/mL)	↓	↑
Plasma PC (%)	↓	↑
Plasma TAG (μg/mL)	↑	↑
Plasma TAG (%)	↓	↑
Plasma CE (μg/mL)	↑	↑
Plasma CE (%)	↔	↑
Plasma NEFA (μg/mL)	↑	↑
Plasma NEFA (%)	↑	↑
PBMC	↓	↑
Placenta	-	↑
Cord tissue	-	↑

4.4 Discussion

4.4.1 Discussion of the current findings

This is the first study to report the effect of increased salmon consumption in pregnant women upon plasma and cell fatty acid concentrations. Changes in fatty acid compositions of maternal lipid pools might be of importance to the woman's own health but will be especially important in terms of the ability of the woman to provide key fatty acids to her developing foetus. The main findings of the work presented in this chapter were:

The hypotheses being examined in this chapter are that:

- increasing habitually low intake of oily fish (≤ 2 portions per month) to 2 portions per week during pregnancy, will increase maternal plasma lipid levels of EPA and DHA, and that these levels will be higher than in the control group;
- the salmon intervention will increase maternal peripheral blood mononuclear cell (PBMC) levels of EPA and DHA, and that these levels will be higher than in the control group;
- the salmon intervention will result in higher placenta and umbilical cord tissue EPA and DHA levels compared to the control group.

It is evident from the data presented that all three hypotheses can be accepted.

At baseline (20 weeks gestation), the salmon group had significantly higher percentages of EPA, DHA, and total n-3 PUFA, and a lower percentage of ARA in plasma PC and in the other plasma lipid fractions, compared to the control group. Oily fish and fish oil supplements are the major dietary sources of LC n-3 PUFA that these have a major influence on the LC n-3 PUFA status in plasma, cells and tissues. In the current study, subjects were not consuming fish oil supplements (see chapter 3). This baseline difference in fatty acid status between the groups probably reflects a baseline difference in fish consumption. Specifically, as discussed in chapter 3, it was found that the salmon group consumed significantly more frequently total fish and shellfish, oily fish, and non-oily fish at 20 weeks gestation, although the subjects were randomly assigned to the study groups. This information was obtained from the FFQ which reflected dietary intake in the past three months. Plasma lipid fatty acids reflect fairly short-term changes in the diet since they are associated, mainly, with continuous lipoprotein synthesis from the liver. However, cell fatty acid composition (especially red blood cells but also mononuclear cells) is affected in the long-term because of the long period of life of the cells (red blood cells

have a half life of about 4 months). There were no baseline differences in the PBMC fatty acid composition between the groups which may reflect that the salmon group consumed more fish closer to the start of the intervention (20 weeks) rather than habitually for a longer period. The baseline differences between the two groups may be considered as a limitation of the current study. For this reason between group comparisons of the change in the fatty acid content between time points (34 minus 20 weeks, and 38 minus 20 weeks), along with the 3 repeated measures ANOVA, were conducted.

Although baseline differences were observed for percentage levels of certain fatty acids, there were no baseline differences when plasma fatty acids were expressed as absolute concentrations (μg/mL plasma). Also, the effects of pregnancy and of the salmon intervention on maternal plasma fatty acid status did not always give the same result when comparing the two different types of expression for the same fatty acid. These two different ways of expressing fatty acid content is an important point to be considered when interpreting the data. Percentages reflect the level of each fatty acid relative to the rest of the fatty acids in the lipid fraction studied. This means that a significant increase in the percentage of one fatty acid will be compensated by a decrease in the percentage of at least one other fatty acid. On the other hand, the absolute concentration of each fatty acid is not relative to the concentration of the rest of the fatty acids, and thus a change in the concentration of one fatty acid is not necessarily followed by a change in that of others. What is more, it is possible that the absolute concentration of a fatty acid increases but its relative percentage remains constant or decreases, and vice versa. For example, it was observed that plasma CE DHA concentration increases whereas percentage DHA in plasma CE remains unchanged during pregnancy in the control group. Also, plasma TAG DHA concentration increased but TAG DHA percentage decreased during pregnancy in the control group. The inclusion of both expressions of fatty acid status in the current study is a strength of the current study as there is no indication for any expression being more advantageous than the other. The combination of the two expressions gives a better picture of the changes that occur. Also, it has to be pointed out here, that the current analysis does not take into account the pregnancy-associated plasma volume expansion.

The calculation of absolute fatty acid concentrations as performed here, allows for total fatty acid absolute concentrations in each lipid class to be determined. It was observed that pregnancy resulted in increased total plasma concentrations of all lipid fractions (especially for CE and NEFA), apart from the PC fraction which increased in concentration initially (between 20 to 34 weeks) and then decreased (at 38 weeks). The hyperlipidemia of pregnancy has been well established in literature (245, 255) and was seen in both groups in

the current study without being affected by the salmon intervention. The increased concentration of lipids in maternal plasma during pregnancy, especially of NEFA and TAG, are important for ensuring supply of fatty acids to the foetus. The increased lipid concentration in maternal plasma results in the required concentration gradient at the materno-foetal interface to allow for fatty acids to be transported via the placenta.

Apart from the changes observed in total fatty acid concentration in each lipid fraction, there were also changes in specific fatty acids associated with pregnancy which were observed in the control group. In total plasma, ARA, EPA, DPA and DHA concentrations decreased during pregnancy. More specifically, changes were also observed with each lipid fraction. ARA, EPA, DPA and DHA concentrations decreased in plasma PC whereas they increased in plasma NEFA, and either increased or remained stable in CE and TAG. On the other hand, ARA, EPA, DPA, and DHA expressed as percentages decreased in all lipid fractions apart from the NEFA fraction where they increased. These results suggest that the mother is progressively depleted in these fatty acids (especially DHA and ARA) during pregnancy as they are transported to the foetus to be used for foetal growth, visual and neuronal development (245, 251). The decrease in EPA is also associated to the fact that it is metabolised to DPA and eventually to DHA which is highly demanded by the foetus (47, 245, 251). The current findings agree with existing literature that the concentrations of ARA and DHA increase during pregnancy but that their percentages in various plasma lipids decrease (260, 265, 270, 271). The pregnancy associated LC n-3 PUFA and ARA depletion observed in the control group must also be related to its very low intake of fish and thus of EPA and DHA. Low intake of these LC n-3 PUFA results in remarkable depletion of these fatty acids during pregnancy and may lead to alterations in the function of the maternal immune system (7, 47, 121).

The current study showed for the first time that consumption of two portions of salmon per week from week 20 of pregnancy until delivery reversed the pregnancy associated decrease in plasma EPA, DPA and DHA, resulting in an increase of these fatty acids in all plasma lipid fractions. The increase of EPA percentages was more pronounced in the plasma PC and NEFA fractions (increase by 20% and 27% respectively). The increase in EPA concentration was more substantial in the TAG and NEFA fractions (120% and 220% respectively). The increase of DHA percentage was greater in the TAG fraction (by 54%). The increase in DHA concentration was greater for the TAG and CE fractions (by 225% and 90% respectively). In contrast, the salmon intervention enhanced the pregnancy associated ARA decrease in maternal plasma lipids. The ratios of fatty acids of interest (ARA/EPA, ARA/DHA, ARA/(EPA+DHA), and n-6/n-3) were lower in the

salmon group compared to control at 34 and at 38 weeks gestation for all plasma lipid fractions. As a generality, the fatty acid ratios decreased in the salmon group during pregnancy, whereas they increased or remained stable in the control group. The data show that increased maternal intake of EPA and DHA through consumption of two portions of salmon per week increased their status in LC n-3 PUFA which was compensated by a lower status of ARA. The lower ARA/DHA ratio in the salmon group may have an impact on the relative transfer of these fatty acids to the foetus via fatty acid transport proteins on the placenta for which these fatty acids may compete (258). Also, fatty acid content in maternal plasma may affect the expression of such placental transport proteins and thus the transfer of fatty acids to the foetus (189). It can be speculated that the increased maternal status in EPA and DHA may have resulted in higher provision of these fatty acids to the foetus which may have beneficial implications for the foetal growth, visual and brain development, and also immune system development. In addition to that, the reversal of the decrease in LC n-3 PUFA may be associated to benefits for the mother herself and for her immune system. The increased provision of LC n-3 PUFA to the foetus is further supported by the fact that consumption of two portions of salmon per week resulted in higher LC n-3 PUFA content in placenta and umbilical cord tissue.

A novel finding of the current study is that pregnancy had an effect on the fatty acid composition of immune cells. Maternal PBMC EPA, DPA, DHA and ARA content decreased during pregnancy. This decrease agrees with the finding that the content of these fatty acids in plasma PC also decreased. Plasma PC fatty acid content is an indication of the cell membrane fatty acid composition and for this reason it is termed as the ‘functional pool’. This was verified by our findings which showed that maternal plasma PC fatty acid correlated very well and significantly with maternal PBMC fatty acid composition. The pregnancy-associated decrease in PBMC LC n-3 PUFA and ARA indicates that immune cells give up these fatty acids in order for them to be transferred to the foetus through the placenta. The current data support that these fatty acids essential for foetal growth and development are mobilised from the maternal cells to plasma lipoprotein CE and TAG fractions, and eventually plasma NEFA, that play a key role in transporting fatty acids to the foetus. Whether the change in fatty acid composition of PBMC is important in influencing (or regulating) pregnancy-associated changes in immune function is not clear, but this is an intriguing possibility since EPA, DHA and ARA all play a role in immune cell function.

It was shown that consumption of two portions of salmon per week not only prevented the pregnancy associated decrease in PBMC EPA and DHA status, but it also resulted in an

increase of PBMC DHA. Moreover, the salmon intervention resulted in further decrease of maternal PBMC ARA content during pregnancy. The decrease of maternal immune cell content in EPA and DHA during pregnancy, and its reversal by the salmon intervention may have an impact on the function of the immune cells, including cytokine and eicosanoid production, which will be further investigated in chapter 5.

A limitation of the current study may be that for PBMC, placenta and cord tissue total lipid fatty acid composition was analysed without separating in lipid fractions. This might not have had much impact on the PBMC results as the majority of the cell fatty acids are in the cell membrane phospholipids. However, for placenta and cord tissue there might have been more specific changes within the different lipid fractions.

Consumption on two portions of salmon per week during pregnancy resulted in higher EPA, DPA, and DHA content in maternal plasma NEFA. This is of great importance as the plasma NEFA fraction is an indication of the fatty acids released by hydrolysis of TAG in adipose tissue. Thus, a higher content of LC n-3 PUFA in plasma NEFA reflects a higher adipose tissue storage of these fatty acids which was achieved by the salmon intervention. This is of high importance not only for foetal supply with LC n-3 PUFA during pregnancy but also for infant supply through breast milk after birth. It has been well established that fatty acids in breast milk originate from maternal adipose tissue fatty acids stored during pregnancy (160, 272). A higher supply of these fatty acids to the infant during the critical window of the initial post-partum period is also vital for its growth and potentially for its immune system modulation.

4.4.2 Comparison with the findings of other studies

4.4.2.1 Fatty acid changes during pregnancy

Otto *et al.* (271) investigated maternal and neonatal fatty acid status in plasma PC in an international study including England. It was found that maternal plasma PC total fatty acid concentration was 1521.97 µg/mL, that of ARA was 136.21 µg/mL, EPA was 8.39 µg/mL, and DHA was 69.68 µg/mL, during early pregnancy (about 14 weeks gestation). Similar concentrations were also reported by Houwelingen *et al.* (264). These concentrations are lower than the concentrations observed in the current study. Maternal plasma was also collected at 22 and 34 weeks gestation in the study of Otto *et al.* (271). It was shown that the total fatty acid concentration as well as that of all fatty acids individually increased during pregnancy. The current study is in agreement with these

earlier observations, since in all four plasma lipid fractions all (or most) fatty acid concentrations increased over the course of pregnancy. Otto *et al.* (271) also showed that, when expressed as percentages, almost all PUFA decreased during pregnancy. This was also observed in the control group in the current study.

Another study (265) compared fatty acids in red blood cell PC during pregnancy (16, 22, 32 weeks) and at delivery in mothers and their neonates with fatty acids in plasma PC. At 22 weeks gestation total fatty acid concentration in maternal plasma PC was 1652 μg/mL, and at 32 weeks it was 1760 μg/mL (again higher than seen in the current study). Absolute concentrations of DHA, total n-3 LC PUFA, and ARA in maternal plasma PC increased during pregnancy. The observations of the current study agree with these findings. In the same study (265) percentage levels of ARA, EPA, and DHA in maternal plasma PC were 8.6%, 0.4% and 3.9% respectively. Percentage levels of ARA, EPA, DPA, and DHA decreased during pregnancy, although they remained stable after 32 weeks gestation. The current study, where percentages of ARA, EPA and DHA in maternal plasma PC at week 20 of pregnancy were approximately 9%, 0.6% and 4.5% generally agree with these earlier values.

Al *et al.* (270) studied the relationship between maternal and neonatal plasma PC fatty acid status. Maternal plasma PC levels were reported every 4 weeks in the period between 10 to 40 weeks gestation. Mean total plasma PC fatty acid concentration increased by 51% between 10 to 40 weeks gestation (1238.11 μg/mL and 1867.84 μg/mL respectively) in maternal plasma PC. Also, the absolute concentrations of individual fatty acids increased during pregnancy, with the greatest increases observed for DHA and AA. The current study found an increase of 40% in total plasma PC from week 20 to week 34 of gestation, and that the absolute concentrations of individual fatty acids increased over this time period. Al *et al.* (270) showed that when maternal plasma PC fatty acids were expressed as percentages, a different pattern was observed. Total n-3 PUFA, total n-6 PUFA and ARA percentages all decreased from 10 to 40 weeks gestation, while percentage DHA increased between 10 to 18 weeks and declined thereafter. In the current study percentage of total n-3 PUFA, ARA, EPA, DPA, and DHA decreased in all lipid fractions apart from the NEFA fraction where they increased during pregnancy in the control group.

The study of De Vriese *et al*. (260) investigated the fatty acid composition of maternal plasma CE and PC during pregnancy and at delivery. Maternal plasma was collected at all three trimesters of pregnancy. In the plasma PC fraction, ARA levels were 8.8%, EPA levels were 0.7% and DHA levels were 5.1% at the second trimester of pregnancy, similar

to values observed in the current study. When comparing levels at first trimester with levels at delivery, plasma PC ARA, EPA and DHA dropped significantly (260). In the current study significant decreases in percentages of ARA, EPA and DHA in plasma PC were observed in the control group. De Vriese *et al.* (260) showed that at the second trimester, mean maternal plasma CE percentages of ARA, EPA and DHA were 6.4%, 0.6%, and 0.8% respectively. In comparison, in the current study the values were about 6.5%, 0.55% and 0.7%. During pregnancy percentages of ARA, EPA and total PUFA decreased in the CE fraction (260), effects that were also seen in the current study.

Berghaus *et al.* (261) compared the fatty acid composition of all plasma lipid classes in maternal and cord blood at delivery. In the maternal plasma TAG fraction, percentages of ARA, EPA and DHA were 0.75%, 0.06%, and 0.32%, respectively. In the current study values for these fatty acids at week 38 of pregnancy were 1.1%, 0.1% and 0.65%, respectively. In the PC fraction, ARA, EPA and DHA were 7.7%, 0.35%, and 2.9% (261) compared with 8.4%, 0.42% and 3.7% at 38 weeks in the current study. In the CE fraction, ARA, EPA and DHA were 5.7%, 0.33%, 0.52% (261). In the current study at 38 weeks these values were 5.5%, 0.38% and 0.61% in the control group. Thus, in general the fatty acid percentages seen in maternal plasma lipids at 38 weeks gestation in the current study are in agreement with the data of Berghaus *et al*. (261) Berghaus *et al.* (261) reported that in the plasma NEFA percentages of ARA, EPA and DHA of 0.74%, 0%, and 0.18%, respectively. In the current study the percentages of these fatty acids in maternal plasma NEFA were higher than these.

Thus, comparing the data from the current study with existing data in the literature indicates that the fatty acid concentrations and proportions seen in maternal plasma lipids at different stages of pregnancy in the control group in the current study are in general agreement with existing data for these fatty acids. As a general rule, the concentrations of fatty acids in maternal plasma increase, whereas the proportions of LC n-3 PUFA and ARA in maternal plasma decrease during pregnancy. The agreement between fatty acid levels in the current study, which was conducted on a UK population of low intake of oily fish, with studies from other North Western European countries (Germany, the Netherlands, Belgium) may imply similar dietary patterns in these ‘western-type’ environments and may also be associated to the similarity of the ethnic groups.

4.4.2.2 Fatty acid composition of placenta

The placenta will have been built up using those fatty acids available from the maternal bloodstream and thus some relationship or similarity between placental and maternal

plasma fatty acid compositions might be expected. Furthermore, placenta fatty acid content might reflect the nutrient supply to the foetus, and its analysis may be a good indicator of placental fatty acid transfer (247, 248). The study of Klinger *et al.* (247) investigated the fatty acid content of placenta tissue lipid fractions in Germany. In placenta PC, ARA, EPA and DHA comprised 24.9%, 0.11%, and 4.72% of fatty acids; in placenta TAG, ARA, EPA and DHA were 15.2%, 0.27% and 4.5%; in placenta CE, ARA, EPA and DHA were 10.6%, 0.45%, and 2.1%; in placenta NEFA, ARA, EPA and DHA were 21.3%, 0.41%, and 4.0%. Similar percentages of these fatty acids in total placenta lipids (as studied here) were reported for UK subjects (16). In the current study percentages of ARA, EPA and DHA in total placental lipids in the control group were 23.9%, 0.18% and 4.9%, in good agreement with the earlier studies in Germany and UK. Placental PC from a Spanish population had higher proportions of EPA (0.40%) and DHA (5.63%) and a slightly lower proportion of ARA (22.6%). These differences might reflect differences in maternal diet including differences in fish intake (273).

4.4.2.3 Maternal fish and n-3 PUFA intake and maternal plasma lipid fatty acid composition

Zhang *et al.* (274) investigated maternal dietary intake of EPA and DHA and levels of these fatty acids in plasma PC of pregnant women and their neonates from river/lake, coastal, and inland regions of China. Dietary intake was assessed using a validated semi-quantitative FFQ which included 156 foods which was administered at 28 and 35 weeks gestation. Median daily intake of EPA in the three regions was 22.4, 28.6, 3.1 mg respectively, and that of DHA was 51.7, 54.7, 33.3 mg, showing that the river/lake and coastal regions had higher intake of these fatty acids, probably from fish and other seafood. The median maternal plasma PC levels of DHA and EPA were higher in the river/lake and coastal regions compared to the inland region. Also, neonates in the inland group had higher levels of ARA and lower levels of EPA and DHA in their plasma PC compared to those in the lake/river and coastal regions. It was concluded that there is a close association between dietary intake of EPA and DHA and the corresponding levels of these fatty acids in maternal plasma PC, and that maternal plasma EPA and DHA were also positively associated with the corresponding levels in cord plasma PC.

4.4.2.4 Fish oil supplementation studies in pregnant and non-pregnant subjects

The study of Damsgaard *et al.* (275) investigated the effects of fish oil and high or low linoleic acid intake on fatty composition of human PBMC. Although this study was conducted on healthy men and not on pregnant women, it provides information about

levels of fatty acids in PBMC. Baseline PBMC percentages of ARA, EPA and DHA were about 22-23%, 0.4-0.5%, and 2.7-2.9%, respectively. In the current study percentages of these fatty acids in PBMC at week 20 of gestation were 18%, 0.6% and 2.4%, respectively. Thus the percentages of EPA and DHA are comparable to those reported by Damsgaard *et al.* (275) but the percentage of ARA is lower in the current study. This may be difference due to sex, to pregnancy or to diet. Damsgaard *et al.* (275) found that fish oil supplementation resulted in higher levels of EPA and DHA, and lower levels of ARA in PBMC, as seen in the current study in the salmon group.

Hawkes *et al.* (276) conducted a trial with DHA-rich tuna oil in healthy mothers from day 3 post-partum. Women were asked to consume daily either placebo, 300 mg DHA plus 70 mg EPA, or 600 mg DHA plus 140 mg EPA. After 4 weeks of supplementation, DHA concentrations in maternal plasma, PBMC, breast milk and breast milk cell PC increased in a linear manner in response to dietary DHA. In PBMC PC in the placebo group, percentages of ARA, EPA and DHA were 23%, 0.43%, and 2.1%; again EPA and DHA were very similar to these values in the current study but ARA was lower. In the high DHA intake group, percentages of PBMC PC EPA (0.56%) and DHA (3.3%) were significantly higher than those of the placebo group. In the current study PBMC EPA at week 34 of gestation was 0.55% while DHA was 2.7%.

In the study of Dunstan *et al*. (180) pregnant women were supplemented with fish oil (4 g/day of fish oil providing 2.2 g/day DHA plus 1.1 g/day EPA) or olive oil as placebo (4 g/day) between week 20 gestation to delivery. At 30 and 37 weeks gestation, fish oil supplementation significantly increased maternal erythrocyte PC EPA and DHA percentages and significantly decreased the percentages of all n-6 PUFA including ARA compared to baseline. The current study did not examine erythrocyte lipids.

In the European multi-center study of Krauss-Etschmann *et al*. (184), pregnant women received a supplement providing 0.5 g/day DHA plus 0.15 g/day EPA or placebo from week 22 of gestation to delivery. Maternal plasma PC DHA levels increased from 5.75% at 20 weeks to 7.49% at 30 weeks and 7.26% at delivery in the fish oil group. Maternal plasma PC EPA levels increased from 0.18% at 20 weeks, to 0.53% at 30 weeks and 0.37% at delivery in the fish oil group. In the current study salmon increased plasma PC EPA and DHA compared with baseline and compared with control, although the increases seen were not as great as those of Krauss-Etschmann *et al.* (184). Krauss-Etschmann *et al*. (184) showed that the intervention also increased DHA levels in cord plasma PC. Cord plasma EPA could not be evaluated as it was below the detection limit. In the same study,

Larque *et al.* (189) showed that fish oil supplementation increased the levels of DHA in placenta PC, whereas ARA levels remained unchanged. In the current study placenta DHA (and EPA) increased, but ARA declined. Also, it was shown that mRNA expression of some fatty acid transport proteins on placenta was correlated with DHA in maternal plasma and placenta PC as well as with DHA in cord plasma PC. Also, placental mRNA expression of membrane lipid carriers was correlated with placenta TAG EPA and DHA and placenta NEFA EPA levels.

4.4.2.5 Increased fish consumption interventions in non-pregnant subjects

In the study of Vidgren *et al.* (243) healthy men where asked to consume five portions of fish per week, or received DHA supplementation (algae or fish derived), or continued their habitual diet for 14 weeks. The subjects kept 4-day food records four times during the study to estimate nutrient intake. It was estimated that in the fish group average intake of EPA was 380 mg/day and that of DHA was 670 mg/day. Increased fish consumption resulted in higher levels of EPA and DHA in plasma lipid pools (PC, TAG, CE) and also in erythrocyte membranes and platelets.

Pot *et al.* (241) studied the effects of increased fish consumption on colorectal cancer markers. The intervention period was 6 months, and subjects were randomly allocated to receive dietary advice plus either two portions per week of oily fish (salmon) (n=82), or non-oily fish (cod) (n = 78), or just dietary advice (n = 82). Serum CE EPA plus DHA increased significantly in the salmon group, whereas in the cod and dietary advice group these did not change. In the salmon group, EPA plus DHA content in serum CE increased from 2.85% at baseline to 3.59% at the end of the intervention, which means that baseline levels of very LC n-3 PUFA increased by about 25%. In the current study, the salmon group EPA plus DHA content in plasma CE was 1.35% (lower than what was reported by Pot *et al.*) and at the end of the intervention it increased to 1.52%. This indicates an increase of 12.5% from the baseline value which is lower than the increase observed in the study of Pot *et al.* (241). The lower levels of CE very LC n-3 PUFA and the lower increase of these levels observed in the current study may be attributed to the maternal supply of these fatty acids to the foetus (whereas Pot *et al.* studied non-pregnant subjects). More importantly, although both Pot *et al.* and the current study supplied two portions of salmon per week, the salmon provided in the study of Pot *et al.* had a much higher content of EPA plus DHA resulting in intake of about 1.4 g EPA+DHA/day which is three times higher than the intake of these fatty acids from the current study salmon (about 490 mg/day).

Din *et al.* (238) examined the effect of increased oily fish intake on platelet-monocyte aggregation. Subjects were asked to consume 500 g of mackerel per week for 4 weeks (providing the equivalent of approximately 1 g of EPA plus DHA per day) or to continue their habitual diet. The dietary intervention with fish resulted in higher percentages of EPA and DHA and lower percentage of ARA in plasma PC at 4 weeks compared to baseline.

The fish intervention studies of Vidgren *et al.* (243), Pot *et al.* (241), and Din *et al.* (238), although not conducted on pregnant women, show a higher level of EPA and DHA in fish consuming subjects and a lower level of ARA compared to control subjects. The current study adds to this body of literature as it is a novel study of fish intervention on pregnant women, and showing that increased oily fish consumption can increase status of LC n-3 PUFA even during pregnancy when the maternal LC n-3 PUFA stores are normally depleted in the benefit of the foetus.

4.5 Summary and conclusions

The available literature indicates that the percentages of EPA and DHA in maternal plasma lipids decline during pregnancy. This was also observed in the control group in the current study and it is probably associated with the conversion of EPA to DHA, and the further preferential transfer of DHA into the placenta which potentially leads to increased provision of DHA to the foetus essential for its development. SIPS is the first randomised controlled trial with fish during pregnancy. The data presented in this chapter show that consumption of two portions of salmon per week between week 20 gestation and delivery not only prevented the pregnancy associated decrease in percentages of EPA and DHA in maternal plasma lipids (and in PBMC), but also resulted in increased levels of these LC n-3 PUFA in all plasma lipid classes and in PBMC, compared to a habitually low intake of oily fish. On the other hand, during normal pregnancy maternal absolute concentrations of fatty acids in plasma increase and this is the characteristic gestational hyperlipidemia which enables the transfer of fatty acids through the placenta to the foetus. This increase was observed in the current study in both of the experimental groups. However, in the salmon group absolute concentrations of EPA and DHA in maternal plasma lipids increased more compared to the control group, resulting in higher absolute concentrations of these fatty acids in the salmon group in all lipid classes. Further, it was shown that the salmon intervention resulted in lower levels of ARA in maternal plasma compared to control. Thus, consumption of two portions of salmon per week from mid-gestation to delivery, which provided at least 403 mg/day EPA plus DHA according to FFQ (or 490 mg/day according to fish diaries – see chapter 3), increased maternal plasma EPA and

DHA levels which could be of significant importance since the mother is not totally depleted and the foetus is potentially provided with greater amounts of these fatty acids. This is further supported by the observations on maternal PBMC and on placenta and cord tissues at birth. This study found, for the first time, that pregnancy is associated with a decline of EPA and DHA in maternal PBMC. The salmon intervention resulted in prevention of this decline for both EPA and DHA, and it further increased the levels of DHA during pregnancy, resulting in higher levels of both EPA and DHA in the salmon group than in the control group at the end of pregnancy. This is of great importance since the functionality of these cells, such as eicosanoid and cytokine production, can be affected by an altered fatty acid composition. Finally, placenta and cord tissue had higher levels of LC n-3 PUFA in the salmon group compared to control. The implications of these results are that increased EPA and DHA concentrations in maternal plasma may facilitate better transfer of key fatty acids to the foetus, and that increased EPA and DHA concentrations in maternal plasma and cells may modulate the immune system of the mother during pregnancy. These effects either alone or together may influence the developing foetal immune system in a way that may reduce the risk of developing atopy.

In summary, pregnancy is associated with changes in the concentrations of blood lipids and in the fatty acid components of these lipids. Provision of oily fish to pregnant women prevents the decline in LC n-3 PUFA seen in plasma lipids and immune cells with pregnancy and results in increased amounts of these fatty acids in plasmas lipids and in immune cells. This may influence of health of the mother and the growth, development and health of the foetus and newborn infant. If pregnant women with low habitual intake of fish consume two portions of salmon per week they will significantly increase their status of EPA and DHA.

5 Salmon in pregnancy study (SIPS): maternal immune system during pregnancy and the effect of salmon intervention

5.1 Introduction

During pregnancy complex immunological changes occur in order to suppress maternal cell-mediated responses to foetal and paternal antigens which allows the normal progression of pregnancy (64). The immunologic balance is shifted towards humoral immunity, and specifically towards type 2 helper T-cell (Th2) responses. This shift happens first in the materno-fetal interface (decidua) and later on in systemic circulation (64, 65). According to Wegmann's hypothesis (277), foetal survival depends on a bias of maternal immune responses towards Th2 immune responses and the inhibition of cytotoxic type 1 helper T-cell (Th1) responses. This has been well demonstrated in mouse models. In humans, pregnancy leads to worsening of clinical symptoms of Th2 dominated diseases (systemic lupus erythematosis) and regression of Th1 diseases (rheumatoid arthritis) (278), consistent with the switch away from Th1-type responses and towards Th2-type responses. Recent studies have challenged the Th2 bias hypothesis of pregnancy (279) suggesting that, although the bias has been observed, it is not clear whether it is essential for successful pregnancy or whether it is secondary to hormonal (progesterone, relaxin, placental growth factor) changes (280). However, in pregnancy-related complications such as recurrent miscarriage and pre-eclampsia, the Th2 bias is not present or it is reversed resulting in Th1 dominance (65). The predominant immune interactions in the decidua are between the placenta trophoblast and maternal natural killer (NK) cells rather than T-cells. Thus, it has been proposed that innate, and not adaptive, immunity controls immunoregulation and type 2 cytokines in normal pregnancy through a shift towards 'type 2 NK cells' (281). Also, the dysregulation of NK cells in peripheral blood and endometrial layers has been associated with reproductive immunopathology and recurrent spontaneous abortions (RSA) (282).

The presence of immunoglobulins (Ig) and cytokines in the amniotic fluid is highly important for the developing foetal immune system. It has been shown that amniotic fluid contains IgE which correlates with maternal circulating IgE levels. Amniotic fluid IgE might bind to $CD23^+$ cells within the lymphoid follicles of the foetal gastrointestinal tract affecting the developing immune system (68). It has been suggested that cytokines present in amniotic fluid (such as IL-16) can be swallowed by the foetus and attract immune cells ($CD4^+$) to the foetal gut. Such observations may signify pathways by which maternal cytokines can affect early immune development (283).

Maternal immune responses during pregnancy have been shown to closely correlate with cytokine production and IgE concentration at birth (284, 285). Healthy neonates

appear to have Th2 polarised allergen-specific responses as a consequence of the maternal Th2 polarisation during pregnancy, and diminished capacity for production of Th1 responses. This is balanced by rapid postnatal maturation of Th1 responses and downregulation of Th2 responses, which continues during the first two years of life with appropriate antigen exposure (66). However, this is not the case for the offspring of atopic mothers. Atopy is characterised by polarisation towards Th2 responses to antigens. This has led to the idea that pregnancy may exacerbate the Th2 biased phenotype of allergic and atopic women (286). It has been shown that allergic women have increased peripheral blood allergen-specific Th2 responses during pregnancy compared to non-allergic pregnant women. It was suggested that the peripheral responses in women with allergic disease may also alter the cytokine milieu in the materno-foetal interface, contributing to altered immune responses of the offspring (287). There is strong evidence that the development of atopy in the offspring is strongly influenced by maternal atopy (288), and that maternal atopy has a greater impact compared to paternal (289, 290).

Prescott *et al.* (291) showed that both atopic and non-atopic infants had a diminished capacity for production of Th1 (IFN-γ) responses. However, it was shown that neonates who subsequently developed atopy initially had reduced capacity of Th2 responses as well. Soon after birth non-atopic infants selectively downregulated their Th2 polarised allergen specific responses. In contrast, atopic infants failed to do, continuing to upregulate Th2 immunity after birth which was believed to contribute to the development of atopy. Moreover, it has been shown that Th1 responses at birth of infants who go on to develop atopy are lower than those of non-atopic children. This ‘immaturity’ of Th1 immunity continues to be evident later on in the first years of infancy (2 years old) for atopic infants versus non atopic (292). Prescott *et al.* (291) showed that skewed cord blood mononuclear cell Th2 cytokine responses (IL-4, IL-5, IL-6, IL-9, IL-3) to common environmental allergens are present in all newborns irrespective of their atopy heritability and are dominated by high level IL-10 (regulatory cytokine) production. It was suggested that the redirection of neonate’s immune responses towards the Th1 cytokine phenotype soon after birth, may be more important that the initial Th2-skewed immunity in explaining the development of atopic disease.

Although elevated cord blood IgE is associated with the development of atopy, asthma, and elevated IgE in childhood, it is thought that cord blood IgE is less valuable than family history in predicting allergy, and it does not have the value of screening test (293). Atopic disease includes allergy, hay-fever, allergic rhinitis, eczema and asthma which are IgE mediated (atopy). Similar responses but which are non-IgE mediated are

termed non-atopic. During allergic sensitisation, environmental antigen (allergen) exposure leads to IgE produced by B-cells which binds to specific receptors on the surfaces of mast cells and basophils. Re-exposure to the same allergen will interact with the bound IgE resulting in histamine and Th2 cytokine (IL-4, IL-5, IL-13) production (42). Especially in early infancy, IgE mediated food allergy and atopic dermatitis can be related to the development of future atopic asthma (294).

Although more than half of the individuals with childhood asthma enter clinical remission by puberty, about half of them develop asthma symptoms again during early adulthood. Thus young adults with childhood asthma that has remitted, continue to be in high risk of future relapse. The mechanism of relapsing asthma in early adulthood remains unclear (295). The occurrence of new-onset asthma during adulthood is common, but there is insufficient understanding of its determinants. A study has recently shown that atopy explains only a small proportion of new-onset asthma (296). Another study showed the importance of exposure to substances in the workplace on new-onset adulthood asthma (297). Another study showed that adult-onset asthma is associated with lifestyle factors such as smoking and increased weight gain. Neither pre-existing atopy nor new atopy measured by skin prick testing was associated with adult-onset asthma (298).

Epidemiological data show that the incidence of childhood atopic disease has increased dramatically over the past three to four decades especially in westernised environments (73, 74, 77). This has coincided with, or followed behind, a decrease in infectious diseases (77). These observations contributed to the development of the so-called hygiene hypothesis, which was initially based upon findings of epidemiological studies which showed that natural microbial exposure may be protective towards atopy development (75-77). The role of adaptive immune responses in this concept has been highlighted by the capacity of microbial antigens to enhance Th1 (IFN-γ) and T-regulatory responses, resulting in a shift away from Th2 responses. However the contribution of decreased microbial antigen exposure in westernised countries to the increasing rates of allergic disease is not clear. It has been suggested that allergic disease may result from an inappropriate balance between T-regulatory and Th2 cells. This imbalance could result from either a deficiency in suppressive activity of T-regulatory cells or strong activation which overcomes regulation (299).

Both adaptive and innate immunity are needed when fighting against infectious microorganisms in humans. These two types of immunity, although involving different types of immune cells and acting with different antigenic specificity, interact and

communicate affecting one another through cytokine production and antigen presentation. The role of innate immunity in explaining the impact of decreased microbial exposure on susceptibility to develop atopic disorders is also important. Pattern recognition receptors, such as CD14 and toll-like receptors (TLR) on monocytes, detect in a non-specific way antigenic molecules on the surface of gram-positive (peptidoglycans, lipoteichoic acid) and gram-negative (e.g. lipopolysaccharide (LPS) recognised by TLR-4). This recognition leads to cytokine production and though complex pathways activates and enhances adaptive immune responses, which may influence the shift away from the Th2 phenotype (see section 1.2 for details on the immune system). Genetic polymorphisms of TLR and CD14 receptor genes have been associated with the pathogenesis of asthma, although this evidence is not clear (300).

Complex multifactorial genetic and environmental interactions determine the development of atopic disease. Recently Martino & Prescott (301) reviewed the evidence that immune development is under epigenetic regulation, including Th1/Th2 cell differentiation, T-regulatory cell differentiation, and Th17 development. It is believed that gene-environment interactions are mediated through epigenetic mechanisms. There is evidence that early environmental exposures are important in activating or silencing genes by altering DNA and histone methylation, histone acetylation, and chromatin structure. As a result, phenotype and disease susceptibility are altered. The evidence suggests that atopy prone individuals have very early developmental differences in innate immune gene expression. Several new studies indicate that *in utero* microbial and dietary exposures can modify allergic disease expression through epigenetic mechanisms. Candidate environmental factors in the allergy epidemic include a reduction in microbial signals promoting Th1/T-regulatory responses (hygiene hypothesis) (75-77), dietary changes, smoking, pollutants (302, 303) and toxins (304). For example, dietary folate as a methyl donor may have the capacity to alter methylation patterns *in utero*. A study in mice showed that folate supplementation during pregnancy, resulted in altered methylation and adversely effected airway and allergic response development (305). One study in humans, has shown that folate supplements in pregnancy are associated with increased childhood wheezing (306).

The role of early dietary exposures and feeding practices in pathogenesis and prevention of allergic disease has been recently reviewed by Jennings & Prescott (307). Food allergen avoidance in allergic disease prevention has been unsuccessful and it is no longer recommended unless allergy is confirmed. A study of maternal egg avoidance during pregnancy showed that rigorous dietary egg exclusion did not eliminate trans-

placental and breast milk ovalbumin passage (308). Dietary factors that the foetus is exposed to *in utero* or at early postnatal stages have shown immunomodulatory effects. These include folate, long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), oligosaccharides, probiotics, vitamin D, retinoic acid, vitamin E (309), and other antioxidants such as selenium. The decreased intake of antioxidant vitamins and selenium (13) are important dietary changes and may be associated with the increased rates of atopy. It has been shown that early childhood wheezing is associated with reduced neonatal and foetal selenium status (92, 213). Also, low maternal vitamin E intake during pregnancy was associated with increased risk of wheezing at 2 years of age (213). The effects of antioxidant intake on allergic disease have been recently reviewed by Allan *et al.* (310). A small number of cohort studies reported potential beneficial associations between maternal intake of antioxidants during pregnancy and childhood asthma. However, there is very limited data on childhood atopic dermatitis and atopic rhinitis. The literature available is too limited to draw firm conclusions on the effect of antioxidants on atopy. Maternal vitamin D intake during pregnancy has been associated with decreased risk of sensitization to food allergens in the offspring at five years old in a prospective cohort study in Finland (209). Other studies also support the importance of vitamin D in atopic disease development (210-212).

Most importantly in the context of this thesis, decreased intake of LC n-3 PUFA accompanying increased intake of n-6 PUFA is a major dietary change which has happened in the last 30-40 years, and may be associated with the increase in childhood atopic disease. The major dietary n-6 PUFA is linoleic acid (18:2n-6) and it can be further metabolised to arachidonic acid (ARA; 20:4n-6). On the other hand, α-linolenic acid (18:3n-3) is the precursor of the LC n-3 PUFA, eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) which are found in oily fish such as salmon and in fish oils. LC PUFA have immunomodulatory properties which differ between the n-3 and n-6 family and are mainly associated with the lipid mediators produced by these fatty acids.

Arachidonic acid is the principal substrate of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes giving rise to 2-series prostaglandins (PG) and thromboxanes (TX) or 5-hydroxy-eicosatetraenoic acids (5-HETE) and 4-series leukotrienes (LT). These mediators, specifically PGE and 4-series LT (LTB_4), have been shown to be important in inflammation and immunity (see section 1.4 for details). For example PGE_2 induces fever, pain and production of inflammatory cytokines. PGE_2 also influences cell-mediated immunity through inhibition of T-cell proliferation, inhibition of

production of Th1 type responses (IL-2, IFN-γ), promotion of Th2 type responses, and induction of IgE production by B-cells (103). Consumption of fish oil results in increased amounts of EPA and DHA in inflammatory cells, partly in the expense of arachidonic acid (106, 311). Changes in the fatty acid composition of immune cells can effect membrane structure, function, fluidity, and that of lipid rafts. As has been reviewed (38, 94, 312), altered fatty acid composition of cell membranes can also alter receptor expression, signal transduction pathways, transcription factor activation, and patterns of gene expression. Most importantly LC n-3 PUFA can alter the production of lipid mediators. The decrease in arachidonic acid results in lower production of eicosanoids derived by this (PGE_2, LTB_4) (313, 314). EPA antagonises ARA and becomes a substrate for COX and LOX enzymes giving rise to 3-series PG, TX and 5-series LT and 5-HETE. These EPA-derived eicosanoids are less potent mediators than those derived from arachidonic acid. Moreover, EPA and DHA give rise to potent anti-inflammatory mediators termed resolvins, docosatrienes, and protectins. The EPA and DHA mediators produced are the main reason why LC n-3 PUFA in fish oil have been characterised as anti-inflammatory. Through their above actions (membrane fluidity, cell signalling and mediator production), EPA and DHA also influence innate immune processes such as phagocytosis and cytokine production by monocytes and macrophages, and influence adaptive immunity altering cytokine production and proliferation of T-cells (see section 1.4 for details). Both *in vitro* and animal feeding studies showed that LC n-3 PUFA inhibit T-cell proliferation and production of IL-2 and IFN-γ (126, 128, 311, 314). However, the results of human supplementation studies indicate that, although high levels of fish oil supplementation may inhibit T-cell responses (113), lower doses do not exhibit significant effects (105, 132). The evidence that fish oil supplementation may affect T-cell function independently of eicosanoid production is conflicting and further studies in humans are needed in order to be able to draw clear conclusions.

It has been shown that LC n-3 PUFA status in maternal blood, breast milk and umbilical cord blood is lower in mothers of atopic children (83-86, 92). Prescott & Dunstan (93) have reviewed the effects of dietary LC PUFA on early immune development and their potential role in immune disease development or prevention. The potential effects of LC PUFA may be of great importance during early life. Potential pathways through which LC PUFA could influence immune programming include effects on: local tissue factors (production of lipid-derived inflammatory mediators, oxidative stress), neonatal antigen presenting cell function, T-regulatory cell function, T-cell function, B-cell function (antibody production), end-organ factors (affecting clinical phenotype).

Epidemiological studies looking at the effect of maternal fish consumption during pregnancy and fish consumption during infancy/childhood suggest that fish consumption may play a protective role against atopic disease development in the children. However little is known about the effect of fish consumption on immune functions. It has been shown that fish oil supplementation during pregnancy and lactation resulted in higher provision of LC n-3 PUFA to the offspring and so in a higher LC n-3 PUFA status in the offspring (22, 177-188). Early fish oil provision was associated with immunologic changes in cord blood which may be consistent with decreased risk of atopy in the offspring and such changes may persist. These studies suggested clinical effects of early fish oil provision including reduced sensitization to common food allergens and reduced prevalence and severity of atopic dermatitis in the first year of life, again with a possible persistence until adolescence with a reduction in eczema, hay fever, and asthma. Also, studies of fish oil supplementation during infancy or childhood have shown protective effects, although the evidence is inconsistent.

There are no published intervention trials with fish during pregnancy. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish (in this case farmed salmon) in pregnant women. 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. As described in Chapter 2, a total of 123 pregnant women were randomly assigned to either consume 2 portions of farmed salmon per week from week 20 of pregnancy until delivery or to continue their habitual diet low in oily fish (less or equal to 2 portions per month). The aim of the work described in this chapter is to investigate the effect of the intervention on the immune system of the women enrolled in SIPS during pregnancy. It is considered that maternal immune system changes during pregnancy will influence the developing immune system of the foetus so affecting its maturation and polarisation in a way that would decrease the risk of developing atopy. The primary hypotheses examined in this chapter are that:

- increasing habitually low intake of oily fish ($\leq$ 2 portions per month) to 2 portions per week during pregnancy, will result in altered maternal immune cell phenotypes during pregnancy;
- the salmon intervention will result in altered maternal innate and adaptive immune responses, measured as Th1, Th2, and regulatory cytokine expression by mononuclear cells cultured with various stimulants;
- the salmon intervention will result in lower production of PGE_2 by mononuclear cells cultured with various stimulants.

5.2 Study design, materials and methods

5.2.1 Study design, subjects, subject characteristics and the intervention

In chapter 2 the study design, the recruiting procedure, the nature of the women recruited and their characteristics, and the nature of the intervention were all described in detail. One group of pregnant women (n = 61) did not undergo any dietary intervention and maintained their habitual diet which was low in oily fish. A second group of pregnant women (n = 62) consumed two portions of farmed salmon per week from week 20 of pregnancy until delivery, which corresponds to the recommended oily fish intake for pregnant women in the UK (1-2 portions per week) (11). Full details of the salmon are given in section 2.4: salmon provided 1.73 g EPA plus DHA per portion (i.e. 3.46 g per week or 494 mg/day), which would result in intake higher than the UK minimum recommendation of 450 mg/day (11). Also, it has been recommended that DHA intake should be at least 200 mg/day in women of reproductive age (19). Subjects were seen in the fasting state at 20, 32-34 and 38 weeks gestation and blood was drawn to enable fatty acid analysis of plasma and PBMC (chapter 4) and measurement of immunological markers, which is the focus of this chapter.

5.2.2 Maternal blood collection

Plastic 2 mL ethylenediaminetetraacetic acid (EDTA) vacutainer blood bottles and plastic 6 mL lithium heparin (LH) vacutainer blood bottles were obtained from NHS Stores, Southampton General Hospital. Maternal blood collection was performed by an adequately trained nurse or member of the SIPS team. At 20, 32-34, and 38 weeks gestation, blood was collected into one 2 mL EDTA bottle and into three 6 mL LH bottles and stored at room temperature for further analysis. The blood collected in EDTA was used to enumerate the total number of leukocytes and the types of leukocytes. The blood collected in LH was used to assess immune cell subsets and for the preparation of peripheral blood mononuclear cells (PBMC) for future culturing; this preparation was performed within 8 hours of collection.

5.2.3 Analysis of total blood leukocytes

Blood 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.

5.2.4 Analysis of blood immune cell subsets

5.2.4.1 Materials

Mouse anti-human fluorochrome-conjugated antibodies were purchased from Serotec. Mouse PE-labelled anti-human CD127 was purchased from IOTest-Beckman Coulter. FACS lysing solution, cell wash, and cell fix were purchased from BD Biosciences.

5.2.4.2 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 a flow cytometer (FACSCalibur, Becton Dickinson, Oxford, UK) equipped with a 488 nm argon blue laser. Lymphocytes were identified based on characteristic properties of the cells with regard to forward scatter (FSC) and side scatter (SSC). FSC and SSC were set to a linear scale, and 10^4 cells were acquired for all analyses, except of the enumeration of T regulatory cells, where 10^5 cells were collected. CaliBRITE 3 beads (Becton Dickinson) were employed for calibration of instrument settings and the same voltage settings were used for each sample. Compensation for spectral fluorochrome overlap was performed. In all experiments, gates were set so that isotype control staining consistently yielded < 1% positive cells. Data were analysed using CELLQuest Pro software (Becton Dickinson). Figure 5.1, Figure 5.2, and Figure 5.3 show typical profiles for each stain set. Such profiles can be used to identify the percentage of a specific cell

population with a pool of cells. These profiles also generate another parameter, median fluorescence intensity (MFI), which is an indication of how dense the marker is on the surface of the cells identified; MFI is related to the level of expression of the marker protein on the cell surface. MFI was determined for the expression of CD127 on T-regulatory cells (Figure 5.3).

Table 5.1. Labelled monoclonal antibodies ("stains") used to identify different cell subsets

Stain	Cell type identified	Phenotype of cell type identified	Volume of stain used (µl)
Negative stain	Control (No stain)		-
Anti-CD3-FITC/Anti-CD4-PE	T helper/inducer cells	$CD3^+CD4^+$	10
Anti-CD3-FITC/Anti-CD8-PE	Cytotoxic/suppressor T cells	$CD3^+CD8^+$	10
Anti-CD3-FITC/Anti-CD16-PE	Natural Killer (NK) cells	$CD3^-CD16^+$	10
Anti-CD3-FITC/Anti-CD19-PE	B-cells	$CD3^-CD19^+$	10
Anti-CD14-FITC + Anti-CD282-PE	TLR-2 bearing monocytes	$CD14^+TLR\text{-}2^+$	10+10
Anti-CD4-FITC + Anti-CD25-PE-Cy5 + Anti-CD127-RPE	T-regulatory cells	$CD4^+CD25^+CD127^{low}$	10+5+20
Anti CD4-FITC + Anti-CD25-PE-Cy5 + IgG1-RPE	T-regulatory cells control		10+5+10
IgG1-FITC + IgG1-PE + IgG1-RPE-Cy5	Triple isotype control		10+10+10

FITC, Fluorescein isothiocyanate isomer 1 (liquid); PE, Phycoerythrin (lyophilised)

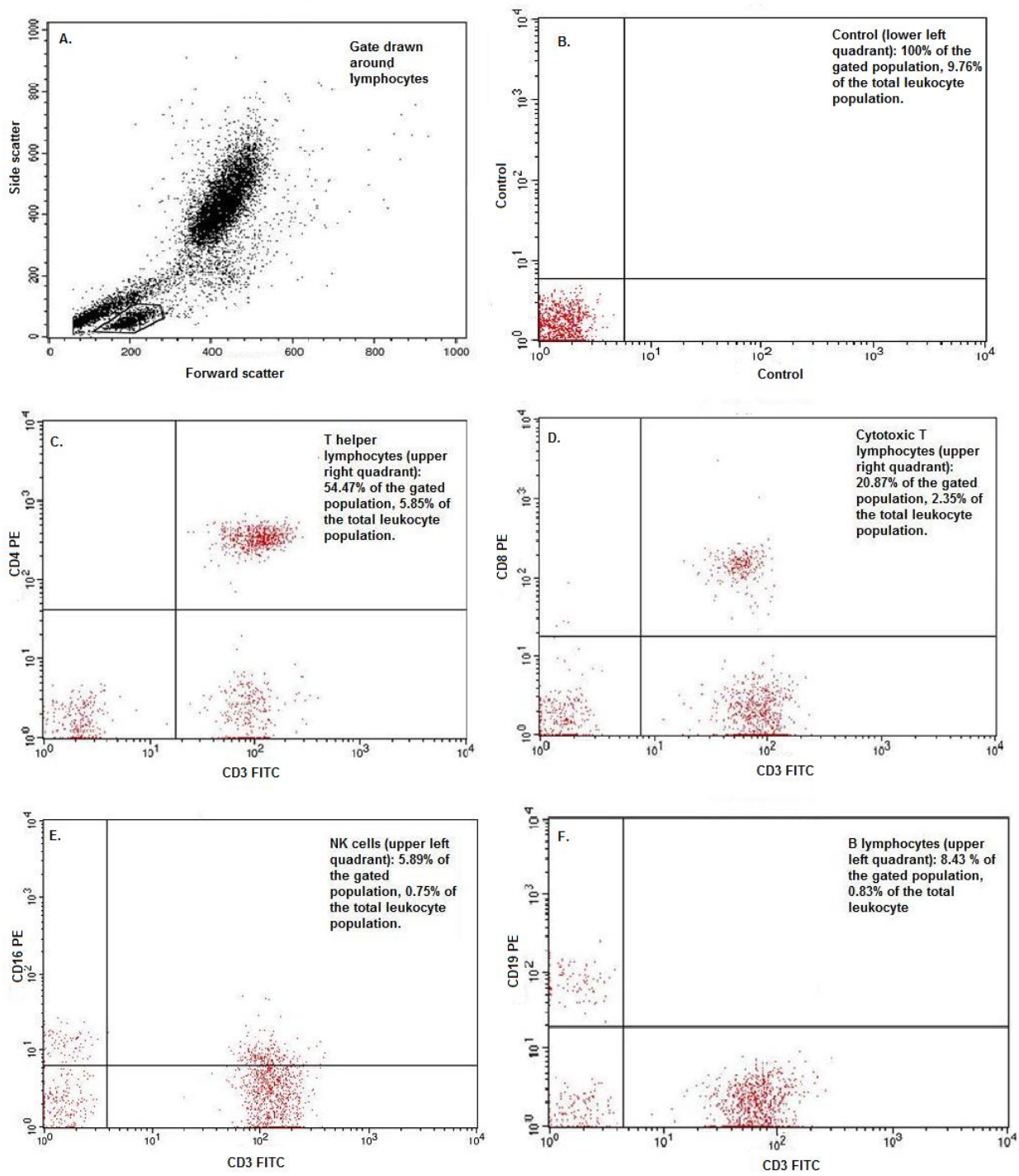


Figure 5.1 Typical flow cytometry profiles of total leukocytes, lymphocytes, and lymphocyte subsets: helper T-cells, cytotoxic T-cells, natural killer (NK) cells, and B-cells

Figures show: gated lymphocytes (A), control (no stain) (B), helper T-cells ($CD3^+/CD4^+$) (C), cytotoxic T-cells ($CD3^+/CD8^+$) (D), natural killer (NK) cells ($CD3^-/CD16^+$) (E) and B-lymphocytes ($CD3^-/CD19^+$) (F)
Figures B-F are based on the lymphocyte gate of figure A.

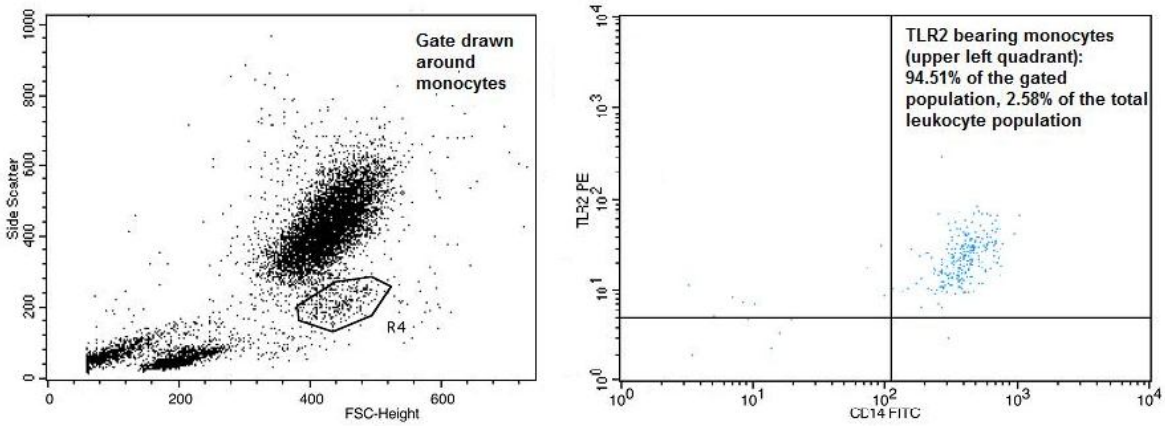


Figure 5.2 Typical flow cytometry profile of monocytes: gated total monocytes (gate R4) and TLR-2 bearing monocytes ($CD14^+/TLR2^+$)

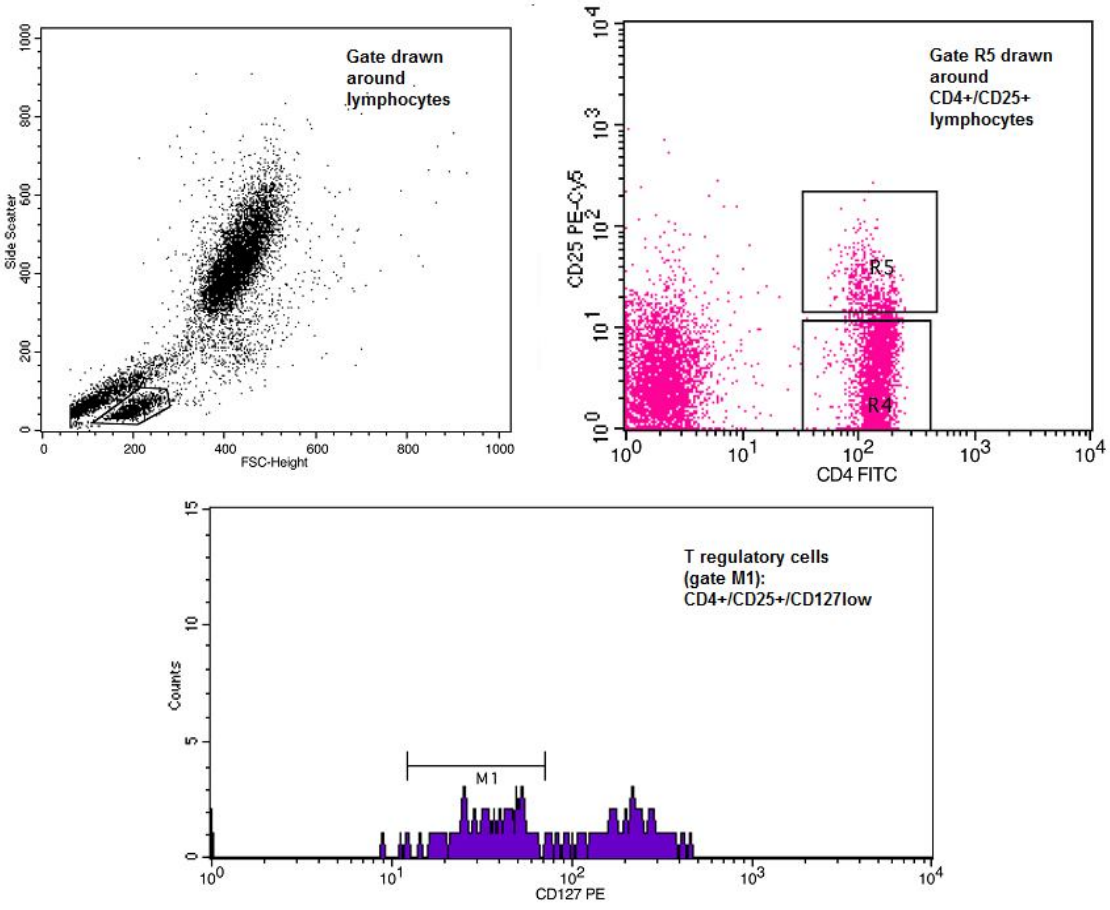


Figure 5.3 Typical flow cytometry profile of T-regulatory cells ($CD4^+/CD25^+/CD127^{low}$)
Gate M1 (low CD127) has been drawn on the $CD4^+/CD25^+$ cells of gate R5
Mean fluorescence intensity was determined for the CD127 receptor

5.2.5 Preparation of peripheral blood plasma and mononuclear cells for culturing

5.2.5.1 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), 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 foetal 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.

5.2.5.2 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 to be used for culturing maternal PBMC and for fatty acid analysis (see chapter 4).

PBMC 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 PBMC number, 2×10^6 cells were removed and frozen at -80˚C for fatty acid analysis (see chapter 4).

The remaining PBMC were washed once (10 min, 450 × *g*) with RPMI medium containing 2% HI-FBS. The PBMC 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 PBMC drop-wise on ice (ratio of resuspended cells in RPMI medium containing 2% HI-FBS to freezing medium: 1:1). The PBMC 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 PBMC were stored in liquid nitrogen for later thawing and culturing.

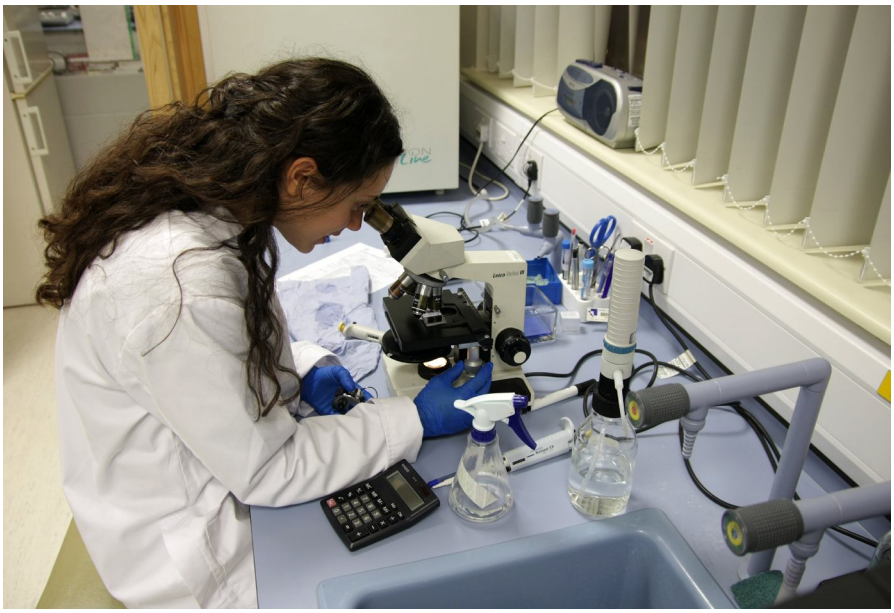
5.2.6 Resuscitation and culture of cryopreserved mononuclear cells

5.2.6.1 Materials

L-glutamine (200 mM), 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 parvalbumin (Sal s1) was a kind gift of John Doe (University of Bergen, Norway).

5.2.6.2 Procedures

Cryopreserved PBMC were thawed rapidly in a 37°C water bath and resuspended with 15 mL ice cold RPMI (containing 0.75 mmol/L glutamine and antibiotics (penicillin and streptomycin)) added dropwise on ice. After centrifugation, cells were resuspended in 1 mL PRMI. Cells were stained with trypan blue and counted using a haemocytometer. Cell viability was assessed and was routinely greater than 80-90%.



All cultures were plated in duplicate in 96-well, round-bottom plates (2 x 10^6/mL) in 200µL of RPMI plus 10% autologous plasma and incubated at 37°C in a 5% CO_2 atmosphere for the times indicated below. The stimuli used were: Der p 1, Sal s1, Ova, PHA, TLR-2 ligand (peptidoglycan (PGN)), TLR-3 ligand (synthetic double stranded RNA Poly I:C), TLR-4 ligand (LPS). Controls wells were left unstimulated. Table 5.2 describes the type, concentration, and amount of each stimulus used. The incubation period for TLR ligands was 24 hours, whereas for allergens and mitogen it was 48 hours. After incubation, the plate was centrifuged and the supernatants were collected and frozen at -20°C for later analysis of cytokines and PGE_2.

Table 5.2 Stimuli used for culturing of maternal peripheral blood mononuclear cells

Stimulant	Type	Final concentration	Volume/well	Amount/well	Culture duration
Der p 1	Allergen	10 µg/mL	10 µL	100 ng	48 h
Sal s1	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

5.2.7 Measurement of cytokine concentrations in mononuclear cell culture supernatant by cytometric bead array (CBA) and flow cytometry

5.2.7.1 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 have been coated with capture antibodies specific for each of the Th1 and Th2 cytokines. The six bead populations are mixed together to form the CBA that is resolved in a red channel (FL3) of a fluorescence-activated cell sorter (FACS). 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.

5.2.7.2 Materials

Human Th1/Th2 cytometric bead array kit purchased from Becton Dickinson Biosciences included human interleukin (IL)-2, IL-4, IL-5, IL-10, tumour necrosis factor (TNF-α), and interferon-γ (IFN-γ) capture beads, human Th1/Th2 PE detection reagent, lyophilised human Th1/Th2 cytokine standards (all contained in one vial), cytometer setup beads, PE positive control detector, FITC positive control detector, wash buffer, assay diluent.

5.2.7.3 Procedure

The BD CBA protocol was followed. The lyophilised cytokine standard, containing all six human Th1/Th2 cytokines, was reconstituted in assay diluent and 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 capture bead populations were pooled together into a 'mixed capture beads' tube before analysis. Per test conducted (standard, negative control, and samples) 4.5 µL from each capture bead were 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 PBMC 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 in the dark for 3 hours at room temperature. After incubation, 1 mL of wash buffer was added to each tube and the tubes were centrifuged (200 × *g*, 5 min). After discarding the supernatant, the bead pellet was resuspended with 300 µL of wash buffer. The samples were analysed using Fluorescence-Activated Cell Sorter (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 BD CBA protocol. Compensation for spectral fluorochrome overlap was performed. The BD CellQuest software was used for data acquisition and formatting. The sample results were generated in graphical and tabular format using the Becton Dickinson CBA analysis software. The limits of detection (LOD) for IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were 2.6, 2.6, 2.4, 2.8, 2.8, 7.1 pg/mL, respectively. Values below level of detection were set at ½ LOD for all cytokines Figure 5.4 shows typical acquisition template example with the six distinct bead populations in the negative control standard and Figure 5.5 that of a culture supernatant from PBMC stimulated with PHA. The populations of the specific capture beads, having discrete fluorescence intensity characteristics, can be seen on the

figures and are distributed from brightest to dimmest as follows: IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ.

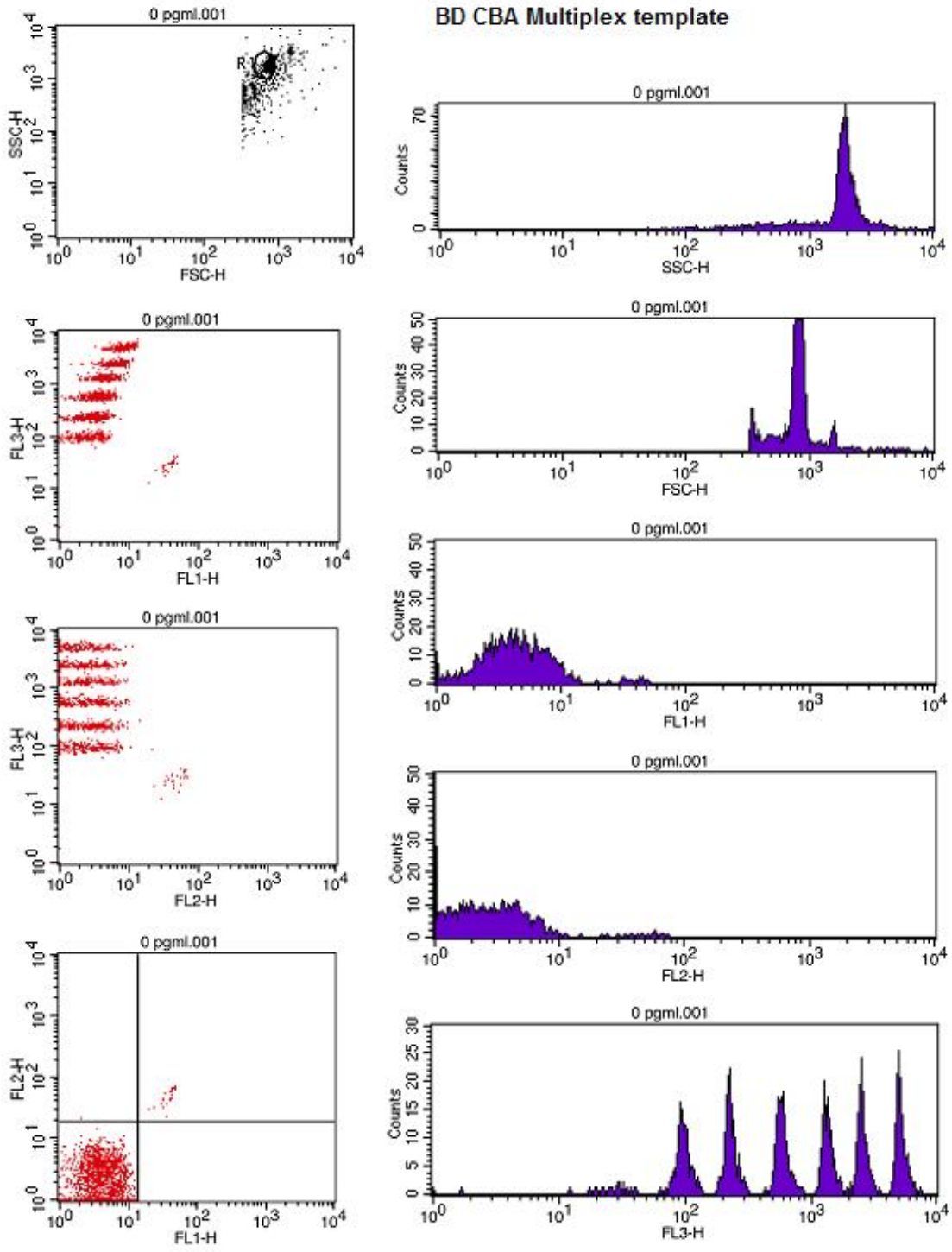


Figure 5.4 Cytometric bead array acquisition template for negative control standard (0 pg/mL)

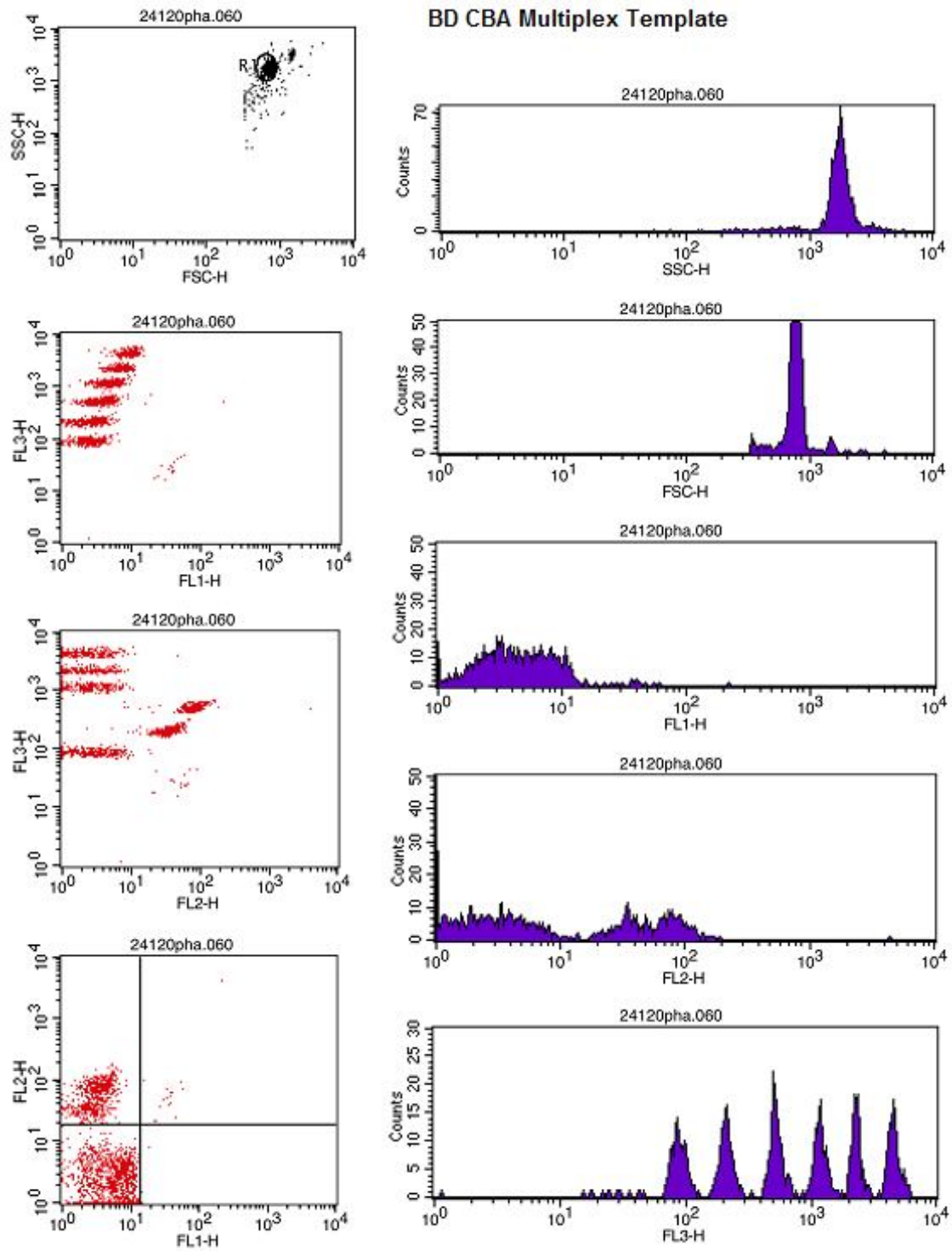


Figure 5.5 Cytometric bead array acquisition template for culture supernatant from PHA stimulated PBMC (48 hours)

5.2.8 Measurement of prostaglandin E_2 concentration in mononuclear cell culture supernatant by enzyme-linked immunosorbent assay

5.2.8.1 Principle

This enzyme-linked immonosorbent assay (ELISA) is based on the forward sequential competitive binding technique 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 antibody sites. Following a wash to remove unbound materials, a substrate solution is added to the wells to determine the bound enzyme activity. The colour development is stopped and the absorbance is read at 450 nm. The colour is inversely proportional to the concentration of PGE_2 in the sample.

5.2.8.2 Materials

PGE_2 ELISA kit purchased from R&D Systems included: goat-antimouse 96 well microplate, HRP-PGE_2 conjugate, PGE_2 standard, primary antibody (monoclonal antibody to PGE_2), calibrator diluent, wash buffer, colour reagent A and B, stop solution

5.2.8.3 Procedure

The procedure followed was according to the manufacturer's protocol. A 1:3, 1:10, or 1:100 dilution of initial culture supernatant was performed as needed in order to be able to detect PGE_2 within the limit of detection. All reagents, working standards, and buffers were prepared according to the protocol. The lyophilised PGE_2 standard was reconstituted with 1 mL distilled water to produce a stock of 25,000 pg/mL. This was left for 15 minutes to stabilise. 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 calibrator diluent was added to the non-specific binding (NSB) wells. 150 µL of calibrator 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 to violet colour apart from the NSB. The plate was again incubated for 2 hours at

room temperature on the shaker. The plates were then aspirated, washed, and blot-dried four times with wash buffer. 200 µL of substrate solution (colour reagents A and B mixture 15 minutes before use according to protocol) 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 each well. The optical density was measured immediately after using a microplate reader (Thermo Labsystems) set at 450 nm. Wavelength correction was set to 550 nm. The software used for plate reading was Ascent (Thermo Labsystems, version 2.6). The NSB optical density was subtracted from that of the standards, control and samples. A standard curve was created for the optical density and PGE_2 standard concentration. PGE_2 concentration corresponding to the absorbance of each sample was calculated from the standard curve. The concentration was multiplied by the dilution factor of the samples.

5.2.9 Statistical analysis

Data on immune cell phenotypes were normally distributed and thus parametric statistical tests were used. The 3-repeated measures analysis of variance (ANOVA) was used as generalised linear modelling to examine overtime changes during pregnancy in relation to the intervention for all immune markers. The independent samples t-test was used for between group comparisons at each of the three time points of measurement (20, 34, 38 weeks gestation). Data on cytokine and PGE_2 expression from cultured PBMC were not normally distributed and the non-parametric 2 independent samples Mann Whitney test was used to compare the two groups at each of the three time points of measurement. The 2 related samples non-parametric Wilcoxon signed rank test was used to compare changes between time points within the groups for not normally distributed data. Pearson's correlation was used for bivariate correlations. The level of statistical significance was set for all tests at p-value ≤ 0.05.

5.3 Results

5.3.1 Maternal leukocytes during pregnancy

Table 5.3 describes the maternal leukocyte counts (cells x 10^3/µL whole blood) during pregnancy in both groups. Repeated measures ANOVA identified significant effects of time on the numbers of total leukocytes, neutrophils, lymphocytes, monocytes, and eosinophils. There were no significant effects of group or group x time interactions. Eosinophils did not change significantly over time in the control group (p for trend = 0.602) but they decreased significantly in the salmon group (p for trend = 0.011). At 20 weeks gestation the salmon group had a lower number of leukocytes compared to the control group.

5.3.2 Maternal mononuclear cell phenotypes during pregnancy

Table 5.4 describes the maternal mononuclear cell phenotypes during pregnancy in both groups expressed as percentage of total lymphocytes, apart from TLR-2^+ monocytes which are expressed as percentage of total monocytes. Repeated measures ANOVA identified significant effects of time on the percentages of B cells ($p < 0.001$) and of group (i.e. salmon vs. control) on the percentage of TLR-2^+ monocytes ($p = 0.026$). There was a significant time x group interaction for the percentage of NK cells ($p = 0.039$). During pregnancy the percentage of NK cells in the total lymphocyte population changed differently in the two groups; it remained stable in the control group (p for trend = 0.425), whereas in the salmon group it decreased significantly (p for trend = 0.002)

The between group comparisons did not reveal differences between the groups at any time point, apart from the significantly higher percentage of TLR-2^+ monocytes in the salmon group compared to the control group at 20 weeks gestation ($p = 0.026$).

Table 5.5 presents the maternal mononuclear cell subsets expressed as percentage of total leukocytes in both study groups. Repeated measures ANOVA identified significant effects of time on the percentages of helper T-cells, T-regulatory cells and TLR-2^+ monocytes and of group (i.e. salmon vs. control) on the percentage of TLR-2^+ monocytes. There was a significant time x group interaction for the percentage of helper T-cells. During pregnancy the percentage of helper T-cells, T-regulatory cells and TLR-2^+ monocytes increased progressively with no significant differences between groups, although there was a tendency for a greater increase in the salmon group. The mean increase of percentage helper T-cells was greater in the salmon group (2.56, ± 2.4) compared to the control group (0.95, ± 2.2) ($p = 0.007$; independent-samples t-test). The

salmon group had a higher percentage of TLR-2^+ monocytes in total leukocytes at 20 and at 34 weeks gestation ($p < 0.001$ and $p = 0.024$ respectively). The MFI of CD127 receptor on T-regulatory cells increased during pregnancy ($p = 0.037$) (Figure 5.6).

Table 5.6 describes the maternal mononuclear cell subset numbers (cells x 10^3/µL whole blood) during pregnancy. These were calculated based on information from Table 5.3 - Table 5.5. Repeated measures ANOVA identified significant time effects on the numbers of helper T-cells, cytotoxic T-cells, T-regulatory cells, and TLR-2^+ monocytes. There were no significant differences between the two groups, apart from the higher number of TLR-2^+ monocytes in the salmon group at 20 weeks gestation.

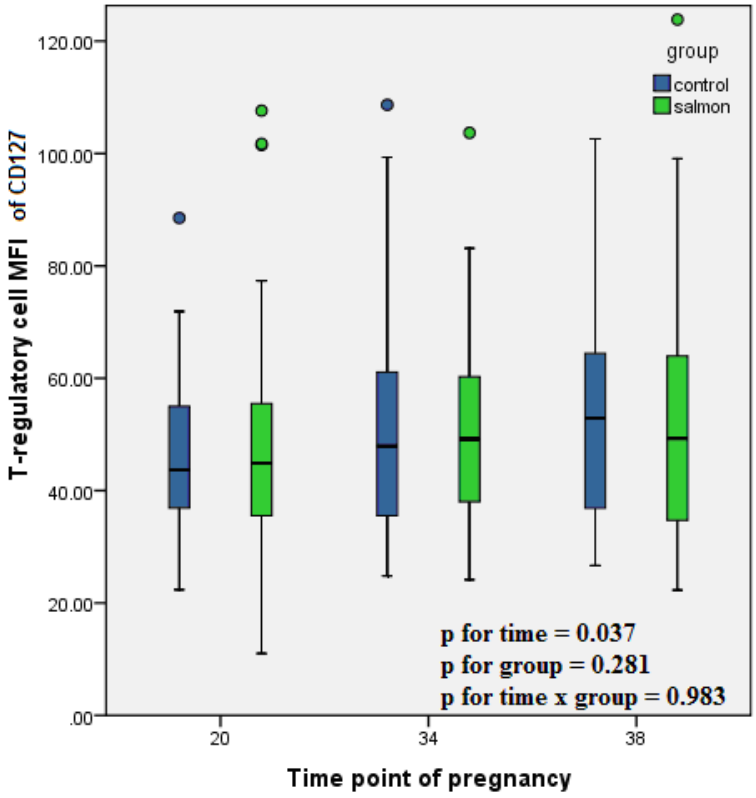


Figure 5.6 Median fluorescence intensity (MFI) of CD127 receptor on T regulatory cells during pregnancy

Table 5.3 Maternal leukocyte counts (cells x 10^3/μL whole blood) during pregnancy

Cell type	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 56	Salmon n = 54	*p**	Control n = 52	Salmon n = 50	*p**	Control n = 37	Salmon n = 44	*p**	Group	Time	Time × group
Neutrophils	6.91 (± 1.8)	6.12 (± 1.6)	0.015	7.11 (± 1.8)	6.62 (± 1.6)	0.150	6.72 (± 1.8)	6.14 (± 1.5)	0.119	0.511	**0.004**	0.839
Lymphocytes	1.59 (± 0.4)	1.49 (± 0.4)	0.165	1.69 (± 0.4)	1.73 (± 0.4)	0.609	1.83 (± 0.4)	1.82 (± 0.5)	0.857	0.829	**<0.001**	0.104
Monocytes	0.49 (± 0.1)	0.52 (± 0.1)	0.208	0.61 (± 0.1)	0.64 (± 0.2)	0.348	0.63 (± 0.2)	0.63 (± 0.2)	0.987	0.433	**<0.001**	0.354
Eosinophils	0.16 (± 0.3)	0.17 (± 0.1)	0.890	0.16 (± 0.3)	0.13 (± 0.1)	0.401	0.18 (± 0.3)	0.12 (± 0.1)	0.298	0.535	**0.01**	0.096
Basophils	0 (± 0)	0 (± 0)	0.430	0 (± 0)	0 (± 0)	0.676	0.02 (± 0)	0.04 (± 0)	0.072	0.260	0.784	0.057
Total leukocytes	9.16 (± 1.9)	8.32 (± 1.8)	**0.022**	9.57 (± 2.1)	9.12 (± 2.0)	0.270	9.37 (± 2.1)	8.72 (± 1.8)	0.136	0.567	**<0.001**	0.669

Values are mean (± standard deviation)
**p*-values from independent samples t-test

Table 5.4 Maternal mononuclear cell phenotypes during pregnancy expressed as percentage of the gated cell population

Cell subsets	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 45	Salmon n = 48	*p**	Control n = 52	Salmon n = 54	*p**	Control n = 41	Salmon n = 47	*p**	Group	Time	Time × group
Helper T-cells ($CD3^+CD4^+$)	48.37 (± 9.07)	49.00 (± 7.82)	0.718	49.02 (± 7.78)	49.57 (± 7.53)	0.712	49.55 (± 7.38)	50.46 (± 8.42)	0.597	0.834	0.108	0.393
Cytotoxic T-cells ($CD3^+CD8^+$)	24.22 (± 6.24)	23.88 (± 6.25)	0.787	24.98 (± 7.10)	24.43 (± 5.99)	0.669	23.57 (± 7.13)	24.42 (± 6.37)	0.557	0.927	0.965	0.191
NK cells ($CD3^-CD16^+$)	6.14 (± 3.72)	7.21 (± 3.12)	0.144	6.14 (± 4.00)	6.36 (± 3.35)	0.767	6.13 (± 3.23)	5.89 (± 3.14)	0.538	0.919	0.127	**0.039**
B-cells ($CD3^-CD19^+$)	10.44 (± 3.62)	10.00 (± 4.33)	0.585	9.73 (± 3.69)	10.10 (± 3.99)	0.618	9.07 (± 3.48)	9.12 (± 3.62)	0.954	0.700	**<0.001**	0.631
TLR-2^+ monocytes ($CD14^+TLR2^+$)	69.80 (± 19.42)	77.31 (± 12.44)	**0.026**	73.45 (± 19.35)	76.24 (± 16.09)	0.423	73.98 (± 20.01)	71.78 (± 16.18)	0.570	**0.039**	0.822	0.428

Values are mean (± standard deviation) percentage of the gated cell population. The cells gated were the lymphocyte population for all cells apart from TLR-2^+ monocytes for which the gated cells were the monocyte population
**p*-values from independent samples t-test

Table 5.5 Maternal mononuclear cell phenotypes during pregnancy expressed as percentage of the total leukocyte population

Cell subsets	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 45	Salmon n = 48	*p**	Control n = 52	Salmon n = 54	*p**	Control n = 41	Salmon n = 47	*p**	Group	Time	Time × group
Helper T-cells ($CD3^+CD4^+$)	6.40 (± 1.82)	6.30 (± 1.77)	0.802	7.10 (± 1.71)	7.76 (± 2.28)	0.097	7.59 (± 2.39)	8.46 (± 2.30)	0.085	0.335	**<0.001**	**0.008**
Cytotoxic T-cells ($CD3^+CD8^+$)	3.17 (± 1.12)	3.36 (± 1.67)	0.510	3.76 (± 1.86)	4.57 (± 5.79)	0.331	3.74 (± 1.43)	4.11 (± 1.67)	0.275	0.196	0.264	0.335
NK cells ($CD3^-CD16^+$)	0.84 (± 0.53)	1.05 (± 0.52)	0.068	0.91 (± 0.58)	0.94 (± 0.54)	0.791	1.01 (± 0.59)	0.96 (± 0.55)	0.711	0.918	0.906	0.120
B-cells ($CD3^-CD19^+$)	1.41 (± 0.66)	1.38 (± 0.73)	0.824	1.61 (± 1.56)	1.54 (± 0.62)	0.738	1.39 (± 0.59)	1.52 (± 0.74)	0.364	0.663	0.273	0.334
TLR-2^+ monocytes ($CD14^+TLR2^+$)	2.17 (± 0.77)	2.88 (± 1.02)	**<0.001**	2.83 (± 1.35)	3.42 (± 1.32)	**0.024**	3.05 (± 1.24)	3.20 (± 1.00)	0.543	**0.002**	**<0.001**	0.432
T-regulatory cells ($CD4^+CD25^+$ $CD127^{low}$)	0.67 (± 0.72)	0.82 (± 0.69)	0.338	1.05 (± 0.62)	1.04 (± 0.61)	0.922	1.16 (± 0.73)	1.20 (± 0.69)	0.824	0.997	**<0.001**	0.765

Values are mean (± standard deviation) percentage of the total leukocyte population

**p*-values from independent samples t-test

Table 5.6 Maternal mononuclear cell subset numbers (cells x 10^3/µL blood) during pregnancy

Cell type	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 44	Salmon n = 40	*p**	Control n = 49	Salmon n = 49	*p**	Control n = 37	Salmon n = 44	*p**	Group	Time	Time × group
Helper T-cells ($CD3^+CD4^+$)	0.757 (±0.2)	0.744 (±0.2)	0.792	0.829 (±0.3)	0.857 (±0.3)	0.591	0.919 (±0.3)	0.919 (±0.3)	0.993	0.206	**<0.001**	0.297
Cytotoxic T-cells ($CD3^+CD8^+$)	0.380 (±0.1)	0.337 (±0.1)	0.137	0.442 (±0.2)	0.424 (±0.1)	0.616	0.429 (±0.2)	0.448 (±0.2)	0.606	0.899	**<0.001**	0.079
NK cells ($CD3^-CD16^+$)	0.095 (±0.1)	0.104 (±0.1)	0.502	0.106 (±0.1)	0.109 (±0.1)	0.782	0.113 (±0.1)	0.106 (±0.1)	0.647	0.799	0.130	0.384
B-cells ($CD3^-CD19^+$)	0.166 (±0.1)	0.143 (±0.1)	0.189	0.171 (±0.1)	0.167 (±0.1)	0.817	0.094 (±0.1)	0.088 (±0.1)	0.949	0.444	0.243	0.470
T-regulatory cells ($CD4^+CD25^+$ $CD127^{low}$)	0.064 (±0.1)	0.079 (±0.1)	0.384	0.107 (±0.1)	0.094 (±0.1)	0.381	0.118 (±0.1)	0.112 (±0.1)	0.760	0.846	**<0.001**	0.512
TLR-2^+ monocytes ($CD14^+TLR2^+$)	0.336 (±0.1)	0.426 (±0.1)	**0.003**	0.456 (±0.1)	0.489 (±0.2)	0.315	0.466 (±0.2)	0.457 (±0.2)	0.823	**0.018**	**<0.001**	0.117

Values are mean (± standard deviation) number of cells x 10^3/µL blood

**p*-values from independent samples t-test

5.3.3 Production of cytokines by maternal mononuclear cells cultured with various stimulants

Concentrations of a range of cytokines (regulatory (IL-10), Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4, IL-5)) in the culture medium of maternal peripheral blood mononuclear cells (PBMC) stimulated with allergens (Der p 1, ovalbumin, salmon paralbumin), mitogen (PHA), and TLR (-2, -3, -4) ligands are presented in Table 5.7-Table 5.10.

IL-10. Production of IL-10 in response to the allergen Der p 1 was low and the concentrations measured were very similar to those seen for unstimulated cells (data not shown). Exposure to the TLR-3 ligand Poly I:C also resulted in low concentrations of IL-10, which were lower than observed for unstimulated cells (Table 5.7). The TLR-2 ligand PGN, the TLR-4 ligand LPS and the mitogen PHA all greatly increased IL-10 production (Table 5.7). Spontaneous production of IL-10 and production in response to and PHA decreased over time in both groups. IL-10 production in response to ovalbumin and to salmon paralbumin decreased in the control group (both p for trend < 0.001) and remained stable in the salmon group (p for trend = 0.201, 0.132). There were no other differences in IL-10 production between groups (Table 5.7).

IFN-γ. Unstimulated PBMC produced very little IFN-γ, although the production that was seen was higher at week 20 of pregnancy, such that there was a significant decrease in concentration over time (Table 5.8). IFN-γ concentration following exposure to the allergens was low and not different from that seen with unstimulated cells (data not shown). PGN, Poly I:C, LPS and especially PHA induced IFN-γ production (Table 5.8). IFN-γ concentration decreased significantly with time for all stimulants with no differences between salmon and control groups (Table 5.8).

TNF-α. Unstimulated PBMC produced TNF-α in readily detectable amounts (Table 5.9). TNF-α concentration following exposure to the allergens was low and not different from that seen with unstimulated cells (data not shown). PGN, LPS and PHA induced TNF-α production, while Poly I:C appeared to suppress it (Table 5.9). TNF-α concentration following exposure to TLR-3 and -4 ligands did not change over the course of pregnancy and was not different between groups (Table 5.9). However, TNF-α production in response to TLR-2 ligand did not change in the control group (p for trend = 0.400) but it decreased and then it increased in the salmon group during pregnancy (p for trend = 0.042). Finally, TNF-α concentration following PHA stimulation decreased over

the course of pregnancy (p = 0.004), although there were no differences between salmon and control groups (Table 5.9).

IL-2, IL-4 and IL-5. IL-2, IL-4, IL-5 concentrations were below the limit if detection in the supernatants of unstimulated PBMC and PBMC stimulated with TLR ligands or with allergens. PHA induced a rise in IL-2, IL-4 and IL-5 concentrations. These decreased over time ($p < 0.001$ for all three; Table 5.10) with no difference between groups.

The ratio of concentrations of IFN-γ to IL-4 in supernatants of cultures of PHA stimulated maternal PBMC was used as a surrogate marker of the Th1 to Th2 ratio and so of the relative activities of Th1 and Th2 cells. This ratio favoured IFN-γ at all time points in both groups and declined over the course of pregnancy, although that was not significant by ANOVA (Table 5.10). However the 2 related samples non-parametric Wilcoxon signed ranks test showed that for both control and salmon groups, the ratio was significantly lower at 34 weeks compared to 20 weeks (p = 0.011 for the control group and p = 0.036 for the salmon group) and at 38 weeks compared to 20 weeks (p = 0.047 for the control group and p = 0.011 for the salmon group). The IFN-γ/IL-10 ratio, which represents the Th1/T-regulatory responses ratio decreased significantly over time (p = 0.001) but was not different between groups. This ratio favoured IFN-γ at 20 weeks gestation and IL-10 at 34 and 38 weeks gestation (Table 5.10).

5.3.4 Cytokine production in relation to skin prick testing

As presented in chapter 2, subjects were skin prick tested with various allergens. It was found that subjects with positive SPT to Der p 1 had higher IL-10 (median 55 vs. 45 pg/mL) and TNF-α (median 11.7 vs. 9.9 pg/mL) production in response to this allergen compared to those with negative SPT, but this was not significant (p = 0.290 and 0.488, Mann Whitney test for all time points and groups combined). The same pattern was observed when data were explored for each time point separately (groups combined), for each group separately (time points combined), and for each time point and group separately (between group comparisons, i.e. salmon vs. control were not significant). The only significant difference found was within subjects with positive SPT to Der p 1, where the salmon group had significantly lower production of IL-10 to Der p 1 compared to control (medians 37 vs. 72 pg/mL, p = 0.012, all time points combined). There were no other allergens used for both ex vivo cytokine production and SPT.

Table 5.7 Interleukin-10 concentration (pg/mL) in supernatants of cultured PBMC collected at different times during pregnancy and stimulated with various TLR ligands, allergens and mitogens for 24 or 48 hours

Stimulant	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 40	Salmon n = 48	*p**	Control n = 42	Salmon n = 49	*p**	Control n = 35	Salmon n = 41	*p**	**Group**	**Time**	**Time × group**
24h control [1]	139.9 (70.7, 261.9)	120.1 (39.6, 188.5)	0.085	78.9 (21.5, 169.1)	54.1 (15.5, 106.7)	0.083	68.8 (37.2, 94.4)	71.1 (26.8, 114.5)	0.799	0.401	**<0.001**	0.223
PGN [1]	439.5 (290.7, 606.6)	409.8 (144.6, 614.1)	0.472	364.6 (218.1, 598.9)	437.5 (207.7, 616.4)	0.656	357.0 (216.4, 504.7)	394.4 (255.4, 577.0)	0.750	0.640	0.406	0.616
Poly I:C [1]	50.8 (16.1, 119.7)	36.4 (7.5, 91.2)	0.313	30.4 (8.4, 82.3)	18.2 (11.9, 53.2)	0.746	28.3 (11.1, 70.1)	20.3 (8.8, 41.8)	0.273	0.793	0.550	0.404
LPS [1]	951.6 (532.5, 1295.0)	860.1 (348.7, 1351.8)	0.586	717.8 (441.5, 1410.3)	815.0 (419.6, 1072.8)	0.971	698.3 (434.7, 1262.2)	940.8 (493.7, 1333.8)	0.527	0.682	0.767	0.776
48h control [2]	89.1 (50.8, 181.1)	71.8 (22.8, 131.5)	0.123	57.1 (23.5, 133.5)	46.8 (21.9, 82.9)	0.324	49.3 (21.9, 121.2)	57.4 (27.8, 99.6)	0.938	0.110	**0.007**	0.384
Ova [2]	114.4 (70.6, 186.7)	74.6 (36.1, 130.2)	**0.016**	67.5 (36.2, 121.5)	58.0 (30.7, 100.0)	0.415	52.2 (29.3, 105.7)	70.0 (36.2, 121.4)	0.479	0.692	**0.004**	0.051
Sal s1 [2]	130.9 (86.3, 188.5)	90.3 (54.3, 149.3)	**0.054**	80.3 (50.3, 135.5)	72.6 (47.2, 115.8)	0.628	73.3 (46.0, 109.7)	80.4 (38.8, 163.5)	0.923	0.663	**0.010**	**0.029**
PHA [2]	180.9 (91.8, 295.4)	128.9 (86.4, 203.0)	0.127	93.2 (34.5, 160.9)	61.9 (31.5, 97.7)	0.077	73.2 (32.8, 143.7)	78.0 (39.8, 117.5)	0.988	0.051	**<0.001**	0.497

Values are median (interquartile range)

The limit of detection (LOD) was 2.8 pg/mL. When the value was below the LOD this was set at LOD/2 i.e. 1.4 pg/mL

**p*-values from non-parametric 2 independent samples test (Mann Whitney)

[1]24 hour mononuclear cell cultures

[2]48 hour mononuclear cell cultures

Table 5.8 Interferon-γ concentration (pg/mL) in supernatants of cultured PBMC collected at different times during pregnancy and stimulated with various TLR ligands and mitogens for 24 or 48 hours

Stimulant	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 40	Salmon n = 48	*p**	Control n = 42	Salmon n = 49	*p**	Control n = 35	Salmon n = 41	*p**	**Group**	**Time**	**Time × group**
24 h control [1]	3.6 (3.6, 12.5)	3.6 (3.6, 8.8)	0.205	3.6 (3.6, 3.6)	3.6 (3.6, 3.6)	0.245	3.6 (3.6, 3.6)	3.6 (3.6, 3.6)	0.516	0.298	**0.029**	0.464
PGN [1]	13.6 (3.6, 29.4)	11.6 (3.6, 39.3)	0.865	3.6 (3.6, 15.9)	3.6 (3.6, 11.8)	0.756	3.6 (3.6, 10.5)	3.6 (3.6, 12.3)	0.838	0.934	**0.037**	0.172
Poly I:C [1]	40.2 (14.8, 274.0)	50.2 (15.3, 148.5)	0.877	10.2 (3.6, 43.9)	16.0 (3.6, 56.2)	0.454	6.9 (3.6, 41.4)	18.5 (3.6, 62.3)	0.067	0.271	**0.004**	0.244
LPS [1]	35.9 (15.1, 91.8)	36.5 (10.9, 102.0)	0.800	14.6 (3.6, 42.0)	18.6 (8.2, 40.0)	0.546	12.3 (3.6, 30.5)	12.9 (3.6, 35.9)	0.441	0.950	**0.004**	0..785
48 h control [2]	8.0 (3.6, 53.2)	3.6 (3.6, 28.5)	0.275	3.6 (3.6, 12.7)	3.6 (3.6, 8.8)	0.675	3.6 (3.6, 5.7)	3.6 (3.6, 3.6)	0.345	0.224	**<0.001**	0.499
PHA [2]	299.2 (90.1, 1011.4)	159.0 (57.5, 1078.1)	0.669	31.9 (6.3, 170.8)	16.6 (3.6, 168.0)	0.386	36.3 (9.7, 201.6)	24.6 (3.6, 163.2)	0.599	0.957	**<0.001**	0.835

Values are median (interquartile range).

The limit of detection (LOD) was 7.1 pg/mL. When the value was below the LOD this was set at LOD/2 i.e. 3.6 (3.55) pg/mL

**p*-values from non-parametric 2 independent samples test (Mann Whitney)

[1]24 hour mononuclear cell cultures

[2]48 hour mononuclear cell cultures

Table 5.9 Tumour necrosis factor-α concentration (pg/mL) in supernatants of cultured PBMC collected at different times during pregnancy and stimulated with various TLR ligands and mitogens for 24 or 48 hours

Stimulant	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 40	Salmon n = 48	*p**	Control n = 42	Salmon n = 49	*p**	Control n = 35	Salmon n = 41	*p**	Group	Time	Time × group
24h control [1]	145.9 (21.7, 274.9)	48.0 (17.3, 166.3)	0.104	63.0 (6.9, 135.9)	19.6 (1.4, 61.6)	***0.054***	21.2 (5.2, 86.5)	18.0 (5.0, 40.0)	0.633	0.923	0.271	0.108
PGN [1]	847.2 (402.4, 1430.9)	722.7 (286.5, 1091.4)	0.090	766.6 (400.0, 1439.6)	784.4 (365.2, 1418.8)	0.676	752.8 (377.8, 1368.3)	677.5 (288.8, 1671.2)	0.977	0.367	0.149	**0.029**
Poly I:C [1]	20.0 (5.4, 60.3)	13.7 (7.4, 58.9)	0.785	10.5 (1.4, 59.0)	7.1 (3.1, 28.3)	0.342	7.0 (3.6, 25.7)	9.2 (1.4, 16.5)	0.790	0.313	0.129	0.537
LPS [1]	643.3 (286.4, 1625.9)	590.5 (224.8, 874.6)	0.283	577.8 (298.6, 825.2)	423.7 (229.0, 930.1)	0.554	465.7 (299.3, 999.4)	540.0 (262.1, 1053.0)	0.590	0.788	0.347	0.193
48h control [2]	26.3 (8.0, 139.3)	25.8 (3.9, 68.9)	0.243	17.0 (1.4, 106.1)	8.2 (1.4, 55.7)	0.428	8.5 (1.4, 42.4)	7.8 (4.0, 20.5)	0.710	0.214	0.541	0.064
PHA [2]	109.7 (30.3, 204.4)	58.3 (24.1, 208.4)	0.325	24.1 (5.0, 82.2)	11.4 (4.5, 83.4)	0.414	21.7 (6.6, 106.3)	16.8 (8.1, 44.6)	0.810	0.956	**0.004**	0.747

Values are median (interquartile range)

The limit of detection (LOD) was 2.8 pg/mL. When the value was below the LOD this was set at LOD/2 i.e. 1.4 pg/mL

**p*-values from non-parametric 2 independent samples test (Mann Whitney)

[1]24 hour mononuclear cell cultures

[2]48 hour mononuclear cell cultures

Table 5.10 Interleukin-2, 4 and 5 concentrations (pg/mL) in supernatants of cultured PBMC collected at different times during pregnancy and stimulated with a mitogen (PHA) for 48 hours

Cytokine	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 40	Salmon n = 48	*p**	Control n = 42	Salmon n = 49	*p**	Control n = 35	Salmon n = 41	*p**	Group	Time	Time × group
IL-2	25.7 (9.3, 37.3)	18.9 (5.6, 49.7)	0.970	6.2 (2.8, 13.6)	5.7 (1.3, 12.5)	0.475	9.7 (3.2, 19.4)	8.7 (2.9, 18.8)	0.810	0.617	**<0.001**	0.667
IL-4	5.1 (1.3, 17.0)	5.5 (1.3, 20.5)	0.691	1.3 (1.3, 3.6)	1.3 (1.3, 2.9)	0.579	1.3 (1.3, 4.1)	1.3 (1.3, 4.9)	0.874	0.776	**<0.001**	0.990
IL-5	6.7 (1.2, 28.5)	7 (2.6, 25.6)	0.619	1.2 (1.2, 5.8)	1.2 (1.2, 4.8)	0.806	1.2 (1.2, 7.4)	1.2 (1.2, 7.8)	0.468	0.654	**<0.001**	0.556
IFN-γ/IL-4	40.7 (13.6, 125.5)	43.6 (8.9, 94.8)	0.615	14.6 (3.4, 47.9)	11.2 (2.7, 50.8)	0.363	12.3 (6.4, 37.2)	8.5 (2.7, 48.4)	0.398	0.528	0.324	0.502
IFN-γ/IL-10	2.2 (0.3, 5.2)	2.1 (0.4, 6.5)	0.828	0.5 (0.1, 1.8)	0.4 (0.1, 3.5)	0.940	0.5 (0.2, 1.7)	0.6 (0.1, 2.1)	0.743	0.226	**0.001**	0.223

Values are median (interquartile range)

**p*-values from non-parametric 2 independent samples test (Mann Whitney)

5.3.5 Production of prostaglandin E_2 by maternal mononuclear cells cultured with various stimulants

Unstimulated PBMC produced significant amounts of PGE_2 and this declined over the course of pregnancy (Table 5.11). At 34 weeks gestation PGE_2 production from unstimulated PBMC was significantly lower in the salmon group compared to the control group (p = 0.023). Exposure to LPS increased PGE_2 production (Table 5.11). PGE_2 production in response to PHA decreased over time but was not significantly different between groups (Table 5.11). PGE_2 production in response to LPS correlated significantly and positively with arachidonic acid content of PBMC (data for both groups and all time points combined) (Figure 5.7). Similarly, significant positive correlations were observed for PGE_2 production from LPS stimulated PBMC and PBMC arachidonic acid content at 20 weeks of pregnancy for both groups combined (Pearson's r = 0.237, p = 0.024), and at 20 weeks for the salmon group (Pearson's r = 0.372, p = 0.011). Also, PGE_2 production from PHA stimulated PBMC correlated negatively with PBMC EPA content for both groups and all time points combined (Pearson's r = -0.144, p= 0.022), and at 38 weeks for both groups combined (Pearson's r= -0.313, p = 0.006). PGE_2 production from unstimulated PBMC correlated negatively with PBMC EPA content at 34 weeks gestation for the salmon group (Pearson's r= -0.347, p = 0.015). Lastly, spontaneous production of PGE2 correlated negatively with PBMC DHA content at 34 weeks for both groups combined (Pearson's r = -0.215, p = 0.038).

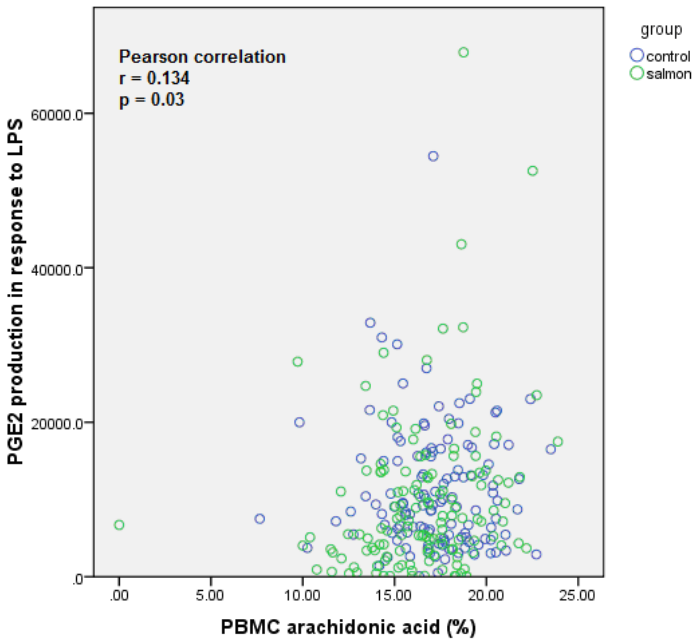


Figure 5.7 Correlation between maternal PBMC arachidonic acid content and PGE_2 production from lipopolysaccharide (LPS; TLR-4 ligand) stimulated PMBC in both groups
All time points during pregnancy included

Table 5.11 Prostaglandin E_2 concentration (pg/mL) in supernatants of cultured PBMC collected at different times during pregnancy and stimulated with TLR-4 ligand (LPS) and a mitogen (PHA)

Stimulant	20 weeks gestation			34 weeks gestation			38 weeks gestation			Repeated measures ANOVA *p*		
	Control n = 45	Salmon n = 49	*p**	Control n = 46	Salmon n = 49	*p**	Control n = 39	Salmon n = 42	*p**	**Group**	**Time**	**Time × group**
Unstimulated (control)	4543.7 (2400.6, 11675.5)	3323 (1362.5, 9030)	0.146	3900 (666.8, 7585.3)	1560 (353, 3854.8)	**0.023**	2812 (1218.4, 4607.7)	1968.5 (603.6, 3550.8)	0.204	0.249	**0.001**	0.486
LPS [1]	9362 (4674-17123)	7151 (3355, 13459)	0.071	9432.1 (5756.3, 15256)	7279.6 (3371.3, 12927.8)	0.098	8604.8 (4897, 13823.7)	6286 (3542.5, 14069)	0.394	0.809	0.224	0.754
PHA [2]	3783 (1741-8955.6)	2595 (1138, 7298)	0.178	2356 (1138, 4808)	2136 (299, 5170)	0.326	2977 (1749, 6272)	2311.1 (1335.5, 4463.3)	0.203	0.200	**0.037**	0.644

Values are median (interquartile range)

**p*-values from non-parametric 2 independent samples test (Mann Whitney)

[1]24 hour mononuclear cell cultures

[2]48 hour mononuclear cell cultures

5.3.6 Summary of the results

Table 5.12 summarises the findings of this chapter and differentiates between the effect of pregnancy and the effect of the salmon intervention. Pregnancy had a significant effect on the number of leukocytes and their subsets. Neutrophil numbers initially increased and then decreased, and lymphocytes, monocytes, and total leukocytes increased during pregnancy in both groups, without any effect of the salmon intervention. Eosinophil numbers did not change during pregnancy but they decreased significantly in the salmon group. During the course of pregnancy, B-cells decreased whereas TLR-2^+ monocytes, T-regulatory cells, and cytotoxic T-cells (only cell number) increased. The salmon intervention had no effect on these. Although NK cells were not affected by pregnancy, the salmon intervention resulted in a decrease in their percentage (% lymphocytes). Helper T-cells increased as a result of pregnancy and this increase was further augmented by the salmon intervention (% leukocytes). PBMC production of cytokines (IL-10, IFN-γ, TNF-α, IL-4, IL-2, IL-5) in response to a mitogen (PHA) declined with the progression of pregnancy. The salmon intervention did not affect this decline. However, IL-10 production decreased during pregnancy also in response to allergens (ovalbumin and salmon paralbumin) and the salmon intervention prevented this decrease. Also, TNF-α production in response to TLR-2 ligand was not affected during the course of pregnancy in the control group, whereas it increased and then decreased in the salmon group. IFN-γ production in response to TLR ligands declined during pregnancy and was not affected by the salmon intervention. Finally, PGE_2 production by unstimulated and mitogen stimulated cells declined over the course of pregnancy. The salmon intervention resulted in greater decline of PGE_2 production from unstimulated cells (34 weeks gestation).

Table 5.12 The effects of pregnancy and of salmon intervention on immune markers

	Effect of pregnancy	Effect of salmon
Neutrophils (cells/µL blood)	↑ then ↓	↔
Lymphocytes (cells/µL blood)	↑	↔
Monocytes (cells/µL blood)	↑	↔
Eosinophils (cells/µL blood)	↔	↓
Total leukocytes (cells/µL blood)	↑	↔
B-cells (%Lymphocytes)	↓	↔
NK cells (%Lympholytes)	↔	↓
Helper T-cells		
% Leukocytes	↑	↑
Cell count (cells/µL blood)	↑	↔
Cytotoxic T-cells (cells/µL blood)	↑	↔
TLR-2^+ monocytes		
% Leukocytes	↑	↔
Cell count (cells/µL blood)	↑	↔
T-regulatory cells		
% Leukocytes	↑	↔
Cell count (cells/µL blood)	↑	↔
Mean fluorescence intensity (MFI)	↑	↔
PBMC IL-10 response to:		
Unstimulated (24, 48 h)	↓	↔
Ovalbumin	↓	prevented ↓
Salmon paralbumin	↓	prevented ↓
Mitogen (PHA)	↓	↔
PBMC IFN-γ response to:		
Unstimulated (24, 48h)	↓	↔
TLR-2 ligand	↓	↔
TLR-3 ligand	↓	↔
TLR-4 ligand	↓	↔
Mitogen (PHA)	↓	↔
PBMC TNF-α response to:		
TLR-2 ligand	↔	↑ then ↓
Mitogen (PHA)	↓	↔
PBMC IL-2 response to mitogen (PHA)	↓	↔
PBMC IL-4 response to mitogen (PHA)	↓	↔
PBMC IL-5 response to mitogen (PHA)	↓	↔
IFN-γ/IL-4 to mitogen (PHA)	↓	↔
IFN-γ/IL-10 to mitogen (PHA)	↓	↔
PGE_2 response to:		
Unstimulated	↓	↓
Mitogen (PHA)	↓	↔

5.4 Discussion

Pregnancy is known to be associated with changes in maternal immune function, believed to be important to prevent rejection of the foetus which could be seen as an allograft (i.e. transplant) that would normally elicit an immune response resulting in its rejection. Thus the immune changes that occur allow a successful pregnancy to ensue. At birth several aspects of the foetal immune response are like those in the mother. Some foetal immune responses indicate a predisposition to atopy. Fatty acid-derived mediators might play a role in the immune changes that occur during pregnancy and in setting up the foetal immune system. In chapter 4 it was shown that increased intake of salmon during pregnancy results in higher n-3 PUFA status in maternal plasma and immune cells. Thus it was thought that salmon intake in pregnancy could influence maternal immunity (studied here) that could impact on development of the foetal immune system and predisposition to atopy. The studies described in this chapter set out to address the hypotheses that increasing habitually low intake of oily fish (≤ 2 portions per month) to 2 portions per week during pregnancy will result in altered maternal immune cell phenotypes during pregnancy, in altered maternal innate and adaptive immune responses, measured as Th1, Th2, and regulatory cytokine expression by mononuclear cells cultured with various stimulants, and in lower production of PGE_2 by mononuclear cells cultured with various stimulants. The results obtained indicate that, while there were some effects of salmon intake on certain immune parameters, these hypotheses cannot be accepted (i.e. increased salmon intake during pregnancy had only a limited impact on maternal immunity, at least as measured here). Furthermore the data obtained show that the effect of progression through pregnancy on immune function is of a much greater magnitude than the effect of salmon: a number of immune measures were significantly affected by progression through pregnancy.

5.4.1 Maternal whole blood immune cell subsets during pregnancy and the effect of increased salmon consumption

The immune cell subset analysis showed that the percentage of B-cells in lymphocytes decreased during pregnancy, whereas the percentage of helper T-cells in total leukocytes (and their total number in the bloodstream) increased during pregnancy. These findings are supported by other studies and are consistent with the immune shifts observed during normal pregnancy (64, 65). The study of Kuhnert *et al.* (315) examined changes in lymphocyte subsets during normal pregnancy and compared these with data from non-pregnant control women. The percentage of B-cells decreased but not-significantly during pregnancy; the percentage of cytotoxic T-cells did not change during pregnancy; the

percentage of helper T-cells increased but not significantly during pregnancy (315). The study of Mahmoud *et al*. (316) showed that helper T-cells (expressed as a percentage of total lymphocytes) were higher in pregnant (44.3%) as opposed to non-pregnant (41.7%) women, whereas the percentage of B-cells was lower in pregnancy (12% versus 13.9%). Madmoud *et al.* (317) showed that the percentage of B-cells was lower and of helper T-cells higher in pregnant than non-pregnant women. The findings of the current study, showing changes in percentages of B-cells and helper T-cells (and their number) from week 20 of pregnancy are in agreement with these comparisons of pregnant versus non-pregnant women. Also, in the current study it was shown that the number of cytotoxic T-cells in blood increased during pregnancy (but not their percentage). Other studies have shown a lower number of these cells in pregnant compared to non-pregnant women (316).

The percentage of T-regulatory cells in total leukocytes (and their number in blood) increased during pregnancy. Also, T-regulatory cell mean fluorescence intensity (MFI) increased during pregnancy which indicates that not only the number of these cells increased but also the level of expression of the cell surface marker CD127 increased suggesting increased activation. This finding is in accordance with previous literature findings on T-regulatory cell pool expansion (i.e. increase in number) during normal pregnancy. Zhao *et al*. (318) showed that foetal alloantigen is responsible for the increase of T-regulatory cells during pregnancy, and the expansion of the T-regulatory population is an important maternal immune response. Taams *et al*. (319) reported that T-regulatory cells ($CD4^{+}CD25^{+}$) play a key role in the suppression and regulation of immune responses, by affecting T-cell responses, antibody production, cytokine secretion, and antigen-presenting cells. It has been suggested that the expansion in the T-regulatory cell pool observed during pregnancy is important for prevention of “transplant” (i.e. foetal) rejection. Tilburgs *et al.* (320) showed that there is a preferential recruitment of foetus-specific T-regulatory cells from maternal peripheral blood to the materno-foetal interface in the decidua, where they may contribute to local regulation of foetus-specific responses.

It should be mentioned that there was a limitation in the method used to identify T-regulatory cells. According to the literature an additional antibody “stain” would more definitively determine $CD4^{+}CD25^{+}CD127^{low}$ cells as T-regulatory cells: this is an antibody to the FOXP3 transcription factor expressed by these cells (321). This was not possible in the current study since the flow cytometer used can detect only three antibody stains simultaneously; using anti-FOXP3 would require detection of four “stains”. Despite this limitation, it seems likely that the cells identified as being positive for CD4, positive for

CD25 and expressing a low level of CD127 (the α chain of IL-7 receptor) are T regulatory cells.

The percentage of $TLR2^+$ monocytes in total leukocytes, as well as their number, increased during pregnancy. This indicates an upregulation of innate immunity during pregnancy which is in agreement with current literature that the innate immune system is important during pregnancy (322-324). During pregnancy the maternal innate immune system responds to the 'semi-allograft' foetus in part by an upregultion of innate immunity. It has been suggested that trophoblast cells regulate this change through an activation via TLR (323). The current findings of an increased percentage and number of $TLR2^+$ monocytes are consistent with this idea.

In the current study the increase in helper T-cells (as a % of leukocytes) was greater in the salmon group compared to the control group. To my knowledge, there is no literature on fish or fish oil intake and maternal immune cell subsets in pregnancy with which to compare this finding. Since an increase in helper T-cells as a % of leukocytes is normally seen during pregnancy, this finding may indicate better progression to successful pregnancy with increased salmon intake.

Also it was found that the percentage of lymphocytes as NK cells (% lymphocytes) decreased in the salmon group but not in the control group. Kuhnert *et al.* (315) showed that percentage of NK cells did not change over pregnancy but was lower compared to non-pregnant women. In contrast Mahmoud *et al.* (316) found no differences between NK cell levels in pregnant and non pregnant women. The regulation of NK cells in peripheral blood and endometrial layers has been associated with reproductive immunopathology and recurrent spontaneous abortions (RSA). Different subsets of NK cells with various functional roles can interact with trophoblasts. An increase in number and/or activity of NK cells and their subsets ($CD56^+/CD16^+$) has been observed in women with RSA (282). This would suggest that a suppression in NK cell numbers and activity would promote successful pregnancy. Thus this effect of salmon may be an important and beneficial one. However, what happens in the maternal periphery may not necessarily reflect what happens in the materno-foetal interface. The only study available with which to compare these findings with NK cells in the salmon group is that of Krauss-Etschmann *et al.* (72) which showed that fish oil supplementation during pregnancy was associated with decreased frequencies of NK cells and $CCR3^+CD8^+$ T-cells in cord blood. However, that study did not investigate the effect of fish oil intervention on maternal immune cell subsets

during pregnancy. There are no previous studies on fish oil supplementation or fish consumption during pregnancy that have reported results on maternal immune cell subsets.

One limitation to the current study is that intracellular markers that would characterise the functional properties of immune cell subsets and differentiate them into Th1 or Th2 phenotypes were not investigated. However, the current study did examine production of Th1 and Th2-type cytokines by unstimulated and stimulated mononuclear cells, a measure that does provide insight into the functional phenotype of the cells present. This work is discussed in the next section. Also, it has to be noted that distinguishing between immunological changes that occur locally in the uterus and systemically in the maternal circulation is important (322). Whether the salmon intervention affected frequencies and/or functionality of immune cell subsets in the materno-foetal interface is unknown and was not investigated here.

5.4.2 Maternal innate and adaptive immune responses during pregnancy

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. The use of allergens as stimulants was important in order to investigate adaptive immune responses of T-cells already sensitised specifically to these allergens. These responses would follow the mechanism of antigen presentation by antigen presenting cells (APC). Only T-cells which have previously encountered these specific allergens and have been activated and developed immunologic memory would respond in culture. The number of T-cells specifically sensitised to each allergen would be low and so longer culture periods were required for such responses to be seen. In fact there was a very poor cytokine response to any of the three allergens used. However, subjects already sensitised to specific allergens were expected to have higher responses. Indeed, subjects with positive SPT to a specific allergen (Der p 1) tended to have higher ex-vivo cytokine production (IL-10, TNF-α) to this allergen compared to those with negative SPT, although this was not significant. Another stimulant which also activates adaptive immune responses is the T-cell mitogen PHA. In contrast to the allergens, the mitogen activates T-cells non-specifically via the CD3 receptor present on their surface. This means that all T-cells respond to the mitogen in a non-specific way and bypassing the need for antigen presentation. The response to mitogen can be used as a positive control since all T-cells are expected to respond non-specifically. Indeed there was a strong cytokine response to PHA. Innate immune system responses were investigated stimulating TLR-2,-3 and -4 with specific ligands. TLR ligands stimulate mainly monocytes which express the different TLR on their surface. TLR stimulation is non-antigen specific, TLR being responsible for recognition of so-called pathogen associated molecular patterns (PAMP). This means that TLR on monocytes recognise a general feature of pathogen structure and respond to this with cytokine production (and phagocytosis if the intact pathogen is present). Cytokines were measured using a flow cytometry based assay termed cytometric bead array, which measures a range of cytokines characteristic for different responses: Th1 (IL-2, TNF-α, IFN-γ), Th2 (IL-4, IL-2) and regulatory (IL-10).

IL-2, IL-4, and IL-5 were only produced in cultures stimulated with PHA; these are prototypical T-cell cytokines and so this observation is consistent with production by T-cells in response to the T-cell mitogen. IFN- γ was strongly produced in response to PHA and weakly in response to the TLR ligands. IFN-γ is a prototypical T-cell (Th1 cell) cytokine so high production in response to PHA accords with this. IFN-γ can also be

produced by monocytes and this explains the weak production when TLR ligands were used. IL-10 and TNF-α were produced in response to PHA and especially the TLR-2 and TLR-4 ligands. Both these cytokines can be produced by T-cells and by monocytes, so this observation is in accordance with this. Interestingly, the TLR-3 ligand decreased production of both IL-10 and TNF-α.

The production of all cytokines determined (IL-10, TNF-α, IFN-γ, IL-2, IL-4, IL-5) by maternal PBMC stimulated with PHA decreased during the course of pregnancy, as did the production of IL-10 in response to allergens. These observations suggest T cell reactivity decreases as pregnancy proceeds. Furthermore, the production of IFN-γ by TLR (-2, -3, -4) ligand stimulated PBMC decreased during pregnancy. The ratio of prototypical Th1 to Th2 cytokines (IFN-γ/IL-4) produced in response to PHA and the ratio of Th1 to T-regulatory cytokines (IFN-γ/IL-10) both decreased significantly during pregnancy. These changes signify a polarisation of the helper T-cells towards the Th2 and T-regulatory phenotype during pregnancy which is in agreement with current literature (277, 278).

Ho *et al.* (325) found that the proportion of IL-4 secreting cells exceeded IFN-γ secreting cells in peripheral blood and in decidua of normal pregnancies (early stages of pregnancy, 6-10 weeks). In the study of Langer-Gould *et al.* (326) $CD4^+$ and $CD8^+$ T-cell subgroups were identified and analysed for simultaneous expression of IFN-γ and CD45RA, IL-2 and CD45RA, TNF and CD45RA, IL-4, or IL-10. They showed that IFN-γ producing $CD8^+$ T-cells decreased during the course of pregnancy. The findings of the current study are consistent with these earlier studies. The findings of the current study also partly agree with those of Breckler *et al.* (287) who investigated the allergen-specific production of IL-10, IL-13, and IFN-γ by PBMC at 20, 30 and 36 weeks gestation and 6 weeks postpartum in allergic and non-allergic pregnant women. For both allergic and non-allergic women, IFN-γ production by stimulated maternal PBMC (house dust mite (HDM), cat hair allergen, and PHA) was lower during pregnancy compared to postpartum. This agrees with the reported Th1 response suppression that has been seen in pregnancy (277, 278) and confirmed in the current study. However Breckler *et al.* (287) reported that (regulatory) IL-10 responses to stimulants did not change during pregnancy and were not different from postpartum levels in either allergic or non-allergic women. This contrasts with the decrease in IL-10 production found in the current study. Finally, Breckler *et al.* (287) showed that Th2 IL-13 response to HDM significantly decreased during pregnancy and were lower in pregnancy compared to postpartum in non-allergic women. This finding agrees with the current finding of decreased Th2 responses (IL-4, IL-5) over pregnancy. However, Breckler *et al.* (287) showed that for allergic mothers IL-13 production in

response to HDM did not change during pregnancy and did not differ from postpartum levels. Allergic women were found to have higher IL-13 responses to HDM compared to non-allergic women both during pregnancy and postpartum. These results show that for both allergic and non-allergic women, although the regulatory responses (IL-10) did not change during pregnancy, Th1 responses (IFN-γ) were suppressed compared to postpartum. Non-allergic women downregulated Th2 responses (IL-13) during pregnancy, while allergic women failed to do so and continued to exhibit high Th2 responses. The authors suggested that these peripheral responses in allergic women may also skew the cytokine milieu at the maternal-fetal interface towards the Th2 phenotype, contributing to increased risk of atopy in the offspring of allergic mothers. This partly agrees with my findings that Th1, Th2, and T-regulatory responses to PHA decreased during pregnancy in both groups.

According to several studies (287, 327, 328) atopy status of the mother may affect maternal cytokine responses during pregnancy which may further affect the materno-foetal interface milieu away from Th1 and towards Th2 responses predisposing the foetus to a higher risk of developing atopy. The SIPS did not differentiate between atopic and non-atopic mothers, but observed a decreased maternal Th2 response. Although cytokine expression from PBMC reflects what happens in maternal periphery and not in the materno-foetal interface, it can speculated that reduced Th2 responses may also be present in decidua. This would result in the foetuses being in low risk of having enhanced Th2 responses at birth and thus lower risk of atopy development. A study (329) investigated maternal unstimulated PBMC cytokine secretion in normal and pre-eclamptic pregnancies. It was found that PBMC from healthy pregnant women secreted higher levels of IL-18 and lower levels of IL-12 compared to those from non-pregnant women. Also the ratio IL-18/IL-12 was significantly higher in healthy pregnant women than non-pregnant women. The Th1/Th2 ratio in PBMC was determined by flow cytometry. It was found that the Th1/Th2 ratio negatively correlated with IL-18/IL-12. It was suggested that elevated IL-18 and decreased IL-12 secretion by maternal PBMC may be involved in the Th2 dominance during normal pregnancy. The Th2 dominance was also observed in the current study (although both Th1 and Th2 responses were suppressed) since the Th1/Th2 ratio at 34 and 38 weeks gestation was significantly lower than at 20 weeks.

In the study of Ho *et al.* (325) after culturing decidual mononuclear cells and maternal PBMC it was found that IL-10 was expressed and exceeded IFN-γ secretion, whereas IL-4 and IL-5 levels were lower than IFN-γ during pregnancy. These findings are in accordance with the current findings on cytokine production from maternal PBMC.

As mentioned in the introduction, the paradox that embryos are not rejected by the maternal immune system despite the presence of paternal MHC histocompatibility antigens has been explained in mice by a Th2 switch at the level of materno-foetal interface. In humans, some hormones enhanced during pregnancy can affect the development of Th1 and Th2 responses. It was found that progesterone promotes the production of Th2-type cytokines (IL-4 and IL-5), whereas relaxin promotes the production of Th1-type cytokines (IFN-γ) by PBMC (280).

There were no major or consistent statistically significant differences between the two groups in terms of cytokine production. IL-10 production from salmon paralbumin stimulated PBMC decreased significantly in the control group whereas it remained stable in the salmon group. The same pattern was observed for IL-10 production in response to ovalbumin. The former observation may be related to the increased salmon consumption of the intervention group which increased exposure of the subjects to salmon antigens. The levels of this regulatory cytokine (IL-10) may be pivotal for the regulation of the immune system of the foetus with increased exposure to salmon antigen through the materno-fetal unit, and may result in better tolerance of the offspring to salmon antigen (i.e. reduced likelihood of mounting an active immune response). Although the study of Breckler *et al.* (287) did not show any difference between during and after pregnancy for IL-10 production, other studies have shown that this cytokine is essential in regulating immune responses in pregnancy and that it is higher in the plasma of pregnant compared to non-pregnant women (330). The only study showing that fish oil supplementation affected maternal T-cell responses is the study of Krauss-Etschmann *et al.* (183). It was shown that fish oil supplementation during pregnancy was associated with lower mRNA expression of IL-1 and IFN-γ in maternal blood at delivery. In contrast, mRNA levels of the regulatory cytokine transforming growth factor-β (TGF-β) were higher in maternal blood at delivery and maternal m-RNA levels of IL-13 and IL-4 did not differ between the groups at delivery. Thus, it appeared that fish oil supplementation during pregnancy downregulates Th1 responses in the mother (183). This agrees with the current observation that the maternal immune system was suppressed and polarised towards Th2/T-regulatory responses during pregnancy in both groups. Warstedt *et al.* (188) conducted a fish oil supplementation trial during pregnancy and lactation. No differences in secretion of chemokines and cytokines (IL-1β, TNF, IL-6, IL-10, IL-12, IFN-γ) were observed in the mothers with fish oil supplementation which agrees with the findings of the current study. In the current study, TNF-α production in response to TLR-2 ligand was not affected in the control group, but it increased and then decreased in the salmon group. This may indicates

a greater upregulation of innate immunity in the salmon group during pregnancy. Since innate immune system is important during pregnancy (322-324) this may indicate a better progression to successful pregnancy in the salmon group.

Although results of the current study indicate that maternal immune responses during pregnancy were suppressed and polarised towards the Th2/T-regulatory phenotype, the hypothesis that increased oily fish intake will alter cytokine expression by maternal PBMC during pregnancy was not supported.

Both *in vitro* and animal feeding studies showed that LC n-3 PUFA inhibit T-cell proliferation and production of IL-2 and IFN-γ (126, 128). The results of human supplementation studies indicate that high levels of fish oil supplementation may inhibit T-cell responses, although lower doses do not exhibit significant effects (105, 132). In fact, one study showed that fish oil supplementation increased IFN-γ production and lymphocyte proliferation in response to concanavalin-A (112). Although fish oil supplementation did not have an effect on IL-4 production, it was shown that EPA concentration in plasma phospholipids were positively correlated with the production of both IL-4 and IFN-γ by stimulated PBMC (112). The same study showed that TNF-α and IL-6 production by mononuclear cells was inhibited after fish oil supplementation (331). A study in healthy men showed that fish oil supplementation reduced IL-6 production from LPS-stimulated whole blood cultures compared to control (olive oil) (332). These studies, although not conducted on pregnant women, show an effect of increased EPA and DHA intake on T-cell function which was not observed in the current study.

Studies of maternal fish oil supplementation during pregnancy or lactation have shown effects on immune markers (including cytokine production in cord blood) in the offspring consistent with decreased risk of developing atopy (22, 178, 183, 185). Also, it has been shown that fish oil supplementation during pregnancy resulted in decreased risk of developing clinical signs of atopy in the offspring (186, 187). Moreover, there are various studies of fish oil supplementation in infancy or childhood showing that fish oil supplementation in early life may affect immune function in a protective way (196, 197, 199), although the evidence is inconsistent (103).

According to the above (22, 178, 183, 185-188, 196, 197, 199), although studies of maternal fish oil supplementation have not reported major changes on maternal T-cell function (apart from the study of Krauss-Etschmann *et al*.), they showed that early fish oil provision is associated with T-cell function changes in cord blood which may be consistent

with decreased risk of developing atopy. Thus, our finding that maternal T-cell responses were not altered by the salmon intervention cannot exclude the possibility that the intervention might have affected foetal T-cell responses and subsequently immune system changes in the offspring. Also, the evidence available suggests that maternal fish oil supplementation may have a protective effect against the development of atopy clinical outcomes in the offspring early in life with a possible persistence until adolescence (103). It may be proposed that immune function changes observed in the offspring are not associated with immune function changes in the maternal blood periphery but in the decidua. Also, production of lipid mediators, such as eicosanoids (as discussed below), and the increased provision of LC n-3 PUFA from the study salmon through the placenta to the foetus may be more important in programming the immune system of the offspring.

5.4.3 Maternal prostaglandin E_2 production from mononuclear cells during pregnancy

PGE_2 production from unstimulated and PHA-stimulated PBMC decreased during pregnancy. This effect was related to the changes in PBMC fatty acid compositions presented in chapter 4. PBMC ARA decreased in both the salmon and control groups (*p* for time < 0.001). ARA from the cell membrane lipids is converted by COX to PGE_2. Thus the effect of pregnancy on PGE_2 production may simply relate to reduced ARA availability. In agreement with this, it was shown that PBMC ARA content correlated significantly and positively with PGE_2 production, whereas there was a significant and negative correlation between PBMC EPA and DHA, and PGE_2 production. Why PGE_2 production in response to LPS was not affected by pregnancy is not clear. To my knowledge there are no previous studies that have looked at PGE_2 production from cultured PBMC during pregnancy. The pregnancy associated reduction in PGE_2 production by unstimulated PBMC occurred more quickly in the salmon group, probably associated with the lower PBMC ARA levels observed in the salmon group compared to the control group. Increased production of PGE_2 by decidual macrophages has been shown to regulate labour initiation at term (333). A study showed that incubation of decidual cells with DHA in culture reduced PGE_2 production, suggesting a mechanism though which fish oil may be protective against preterm birth. In the current study decidual production of PGE_2 was not measured, although it could be anticipated that it would be lower in the salmon group in relation to the observed lower ARA placenta content. Lower PGE_2 production in the placenta could potentially explain the slightly longer, but not significant, gestational length in the salmon group (chapter 2), however further investigation is needed to confirm this. One limitation in further interpreting these results is that other lipid mediators such as PGE_3, LTB_4, LTB_5, resolvins, and protectins were not measured. The determination of other mediators would help in showing that higher EPA and DHA content in PBMC, due to salmon intake, leads to the production of less potent mediators.

In accordance to the current findings, a randomised controlled trial of various doses of EPA showed that EPA was incorporated in a linear dose-response fashion into plasma and mononuclear cell phospholipids. It was shown that increased EPA incorporation into PBMC was associated with decreased production of PGE_2 in response to LPS (313). Another study showed that fish oil supplementation decreased PGE_2 production by stimulated PBMC (112). Warstedt *et al.* (188) conducted a fish oil supplementation trial during pregnancy (from week 25) and lactation. They showed that plasma phospholipid EPA and DHA increased in the fish-oil-treated women during pregnancy. LPS-induced

PGE_2 secretion from whole blood cultures was decreased in the majority of women in the fish oil group (between baseline at 25 weeks gestation and 1 week postpartum). However, the mean decrease in the fish oil group was not significant. In contrast, the PGE_2 production in the control group increased significantly (between 25 weeks gestation and 1 week postpartum). The non-significant decrease of PGE_2 secretion in the intervention group was probably due to the observation of higher secretion at baseline from the mothers that were randomized to the fish oil group compared to those allocated in the placebo group. Most importantly, the change in PGE_2 production differed significantly between the two groups. Also, the decrease in PGE_2 secretion in the fish oil group was more pronounced among non-atopic women (but not significant). No differences in secretion of LTB_4 were observed in the mothers with fish oil supplementation (188). These results party agree with our findings supporting the idea that increased intake of EPA and DHA from fish may result in lower PGE_2 production in the maternal periphery but also in the materno-foetal interface resulting in a less inflammatory environment for the foetus, independently of cytokine production, and thus reduction of the risk of developing atopy in the offspring. The study of Prescott & Dunstan (182) 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 also in the current study, increased provision of EPA and DHA to the foetus will result in decreased production of ARA derived lipid mediators and increased production of the less potent EPA and DHA derived lipid mediators (PGE_3, LTB_5) from the offspring's immune cells. This may affect the offspring's T-cell function and polarisation and it may also predispose to a less inflammatory environment during early life, contributing to lower risk of developing atopy in the offspring related to increased maternal oily fish intake during pregnancy.

5.5 Summary and conclusions

The hypotheses that salmon intervention will result in altered immune cell phenotypes and different maternal innate and adaptive immune responses were not supported. However, a number of changes occurring in both groups consistent with the current literature on the immunology of pregnancy were observed. The percentage of B-cells decreased and that of T-helper cells (and their number) increased during pregnancy. The expansion of T-regulatory cells which plays a key role in successful pregnancy was also observed. In addition, both adaptive and innate immune responses of mononuclear cells were suppressed for all cytokines during pregnancy. Maternal immune responses were polarised towards the Th2 and T-regulatory responses. Interestingly, maternal regulatory (IL-10)

response to salmon paralbumin and ovalbumin remained stable for the salmon group, whereas it decreased for the control group. This may have been associated with the increased exposure to salmon in the intervention group. Immune effects at the materno-foetal interface which may have an important role in immune system development of the foetus were not investigated, and the possibility that the salmon intervention may have resulted in local immune alterations cannot be excluded. The salmon intervention may have resulted in programming of the immune system of the foetus and the developing offspring, mediated, not through changes in maternal immune cell phenotype and function, but through the provision of higher amounts of LC n-3 PUFA and lipid mediator alterations in the decidua. Production of PGE_2 by maternal mononuclear cells decreased during pregnancy in both groups probably associated with the decreased ARA content of those cells, since there was a significant negative correlation between PBMC ARA content and PGE_2 production. However, at 34 weeks gestation PGE_2 expression by unstimulated mononuclear cells was lower in the salmon group. This may be accompanied by increased eicosanoid and lipid mediators from EPA and DHA which have less inflammatory potency. This may potentially affect the offspring's T-cell function and polarisation and it may also predispose to a less inflammatory environment during early life, contributing to lower risk of developing atopy in the offspring related to increased maternal oily fish intake during pregnancy.

6 Final Discussion

Epidemiological data show that the incidence of childhood atopic disease (allergy, hay-fever, atopic dermatitis, atopic asthma) has increased dramatically over the past three to four decades especially in westernised environments (73, 74, 77). This has coincided with, or followed behind, a decrease in infectious diseases which has lead to the idea of the so-called hygiene hypothesis (75-77). Other candidate environmental factors in the atopy epidemic include dietary changes, smoking, and pollutants (302-304). Decreased intake of long chain (LC) n-3 polyunsaturated fatty acids (PUFA) (eicosapentaenoic acid (EPA); 20:3n-3, and docosahexaenoic acid (DHA); 22:6n-3) accompanying increased intake of n-6 PUFA is a major dietary change which has happened in the last 30-40 years, and may be associated with the increase in childhood atopic disease. The decreased intake of vitamin D and of antioxidant vitamins and minerals such as selenium (13) are important dietary changes and may also be associated with the increased rates of atopy. Exposure of the foetus or the newborn infant to LC n-3 PUFA, vitamin D, and selenium has been shown to have immunodomulatory effects (103, 307).

Epidemiological studies provide a fairly strong evidence base that fish consumption by pregnant women is protective against atopy development in their offspring (103). It is recommended that pregnant women and women of reproductive age should consume two portions of fish per week, of which at least one should be oily, in order to achieve a minimum intake of 450 mg/day of EPA plus DHA (11). While it is widely considered that the protective effect of fish is due to the provision of LC n-3 PUFA, fish also provide other nutrients such as vitamin D and selenium. It is possible that it is these nutrients or the combination of these nutrients with LC n-3 PUFA that is important. To examine this further, early intervention with fish is required.

There are no published intervention trials with fish during pregnancy. The Salmon in Pregnancy Study (SIPS) is the first single blind (investigator) randomised controlled trial with oily fish (in this case farmed salmon) in pregnant women. 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 overarching SIPS hypothesis, is 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 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 focused only on part of the SIPS data. The aims of the work described in this thesis were: a) to increase oily fish consumption from a habitually low

intake (≤ 2 portions/month) to the recommended maximum level of intake (two portions/ week) between week 20 gestation to delivery, in women whose offspring is at a risk of developing atopic disease; b) to investigate the effect of increased oily fish consumption on maternal blood and tissue LC n-3 PUFA status; and c) to investigate the effect of increased oily fish consumption on maternal immunity, which may potentially influence the developing foetal immune system and atopy risk of the offspring.

The research hypotheses examined on this thesis were that increased consumption of salmon during pregnancy (week 20 gestation to delivery) by women with high risk of having an atopic offspring will:

d) increase maternal LC n-3 PUFA intake;
e) increase maternal LC n-3 PUFA status;
f) alter maternal immunity, which may potentially influence the developing foetal immune system, in a way that would decrease atopy risk for the offspring

The data obtained allowed the first two hypotheses to be accepted (Chapters 3 and 4), whereas there was limited impact of the salmon intervention on the third hypothesis (chapter 5), and thus it cannot be accepted. The main findings of the present thesis are outlined below and further discussed in the context of the currently available literature.

A total of 123 pregnant women whose baby had a high risk of being atopic and with a self-reported low habitual intake of oily fish were randomly assigned to consume two portions of oily fish (farmed salmon) per week or to continue their habitual diet low in oily fish, from week 20 gestation until the end of their pregnancy. For study design and recruitment details see chapter 2. Briefly, subjects were seen at 20 (n = 61 control group; n = 62 salmon group), 32-34 (n = 110), and 38 weeks of pregnancy (n = 91). Up to delivery, the withdrawal rate was 14.5% for the salmon arm and 11.5% for the control arm, and 13% for the total of the subjects. Birth samples were collected from 101 subjects. A total of 88 (n = 41 control group; n = 47 salmon group) 3 month postpartum appointments were completed.

Main findings from the dietary and nutrient analysis. The salmon intervention was successful in achieving consumption of 2 portions of study salmon per week in the intervention group, whereas the control group maintained their habitually low intake. According to the food frequency questionnaire (FFQ) at 34 weeks gestation all subjects (100%) assigned in the salmon group reported having oily fish 1-2 times per week. The

fish diaries showed that the median consumption frequency of study salmon from the salmon group was 1.94 portions per week. Also, it was shown that, 79.6% of the volunteers in the salmon group were having 2 or more portions of study salmon per week for the 81.25% of intervention period. The control group had a very low intake of oily fish and total fish and this was significantly lower than the salmon group. These data prove compliance to the intervention. The median intake of LC n-3 PUFA from total fish for the salmon group during intervention was 402.6 mg/day EPA plus DHA according to FFQ at 34 weeks (or 490.9 mg/day according to fish diaries), which was significantly higher from that of the control group (30.1 mg/day from FFQ, or 24.4 mg/day from fish diaries). This intake covers the recommendation for minimum intake of EPA plus DHA during pregnancy (108% of 450 mg/day). Also, DHA intake in the salmon group covered more than the minimum recommended intake for DHA (200 mg/day) (19). Interestingly, according to the 34 week FFQ, study salmon intake resulted in higher total diet selenium and vitamin D intake in the salmon group compared to control (selenium 58.2 µg/day vs. 49 µg/day; vitamin D 5.75 µg/day vs. 2.9 µg/day, for salmon vs. control groups).

Main findings on maternal fatty acid status. It was hypothesised that increased provision of LC n-3 PUFA from study salmon will result in increased status of these fatty acids in the mother. SIPS findings agreed well with the findings of previous studies on the pregnancy-associated changes in the concentrations of blood lipids and in the fatty acid components of these lipids (i.e. the changes seen in the control group). Interestingly, it was shown for the first time that pregnancy results in lower content of LC n-3 PUFA in maternal peripheral blood mononuclear cells (PBMC), suggesting depletion of LC n-3 PUFA in this pool during pregnancy. The salmon intervention had a significant effect on maternal EPA, DHA, and DPA status which was differentiated from the effect of pregnancy on fatty acid status. It was shown that the increased intake of EPA and DHA from two portions of oily fish per week prevented the decline in LC n-3 PUFA seen in plasma lipids and immune cells with pregnancy and resulted in increased amounts of these fatty acids in plasma lipids and in immune cells. This could be of significant importance since the mother is not totally depleted and the foetus is potentially provided with greater amounts of these fatty acids. What is more, placenta and umbilical cord tissue had also higher content of LC n-3 PUFA in the salmon group compared to control. The implications of these results are that increased EPA and DHA concentrations in maternal plasma may facilitate better transfer of key fatty acids to the foetus, and that increased EPA and DHA concentrations in maternal plasma and cells may modulate the immune system of the

mother during pregnancy. These effects either alone or together may influence the developing foetal immune system in a way that may reduce the risk of developing atopy.

Main findings on maternal immune system. It was considered that the increased intake of EPA plus DHA during pregnancy which resulted in increased status of these fatty acids in the mother, and especially in maternal immune cells, will have immunomodulatory effects for the mother which could consequently affect the developing foetal immune system in a protective way. The results did not support the hypotheses that the salmon intervention will result in altered maternal immune cell phenotypes during pregnancy, in altered maternal innate and adaptive immune responses, measured as Th1, Th2, and regulatory cytokine expression by mononuclear cells cultured with various stimulants, and in lower production of PGE_2 by mononuclear cells cultured with various stimulants. The results obtained indicate that, while there were some effects of salmon intake on certain immune parameters, these hypotheses cannot be accepted (i.e. increased salmon intake during pregnancy had only a limited impact on maternal immunity, at least as measured here). However, significant alterations of the immune system associated with pregnancy, in line with the existing literature, occurred and were not affected by the salmon intervention.

According to the above SIPS is the first randomised control trial to show that if pregnant women who do not habitually consume oily fish, eat two portions of oily fish (salmon) per week, from week 20 gestation to delivery, they will significantly increase:

- their intake of LC n-3 PUFA (achieving recommended intake), vitamin D, and selenium, and
- their status in LC n-3 PUFA

The increased maternal LC n-3 PUFA status did not result in maternal immune system changes.

These main outcomes along with secondary outcomes described in this thesis will be discussed in the context of existing literature in the following paragraphs.

SIPS intervention was conducted between mid pregnancy to delivery. This period of intervention was chosen based upon previous fish oil supplementation studies which focused on similar periods of gestation indicating that this is a critical window for early life intervention (22, 184, 186, 188). Also, the demand in LC n-3 PUFA increases dramatically during the last trimester of pregnancy (245, 258).

In SIPS, the combination of the FFQ with the fish diary is considered as a strength as it allows control over compliance, and also provides the information required to investigate dietary patterns and nutrient intake and changes in these. Although the SIPS sample size was small, because of the primary interest in fish intake and the long period of intervention, it seemed appropriate to use a FFQ especially developed for use in the Southampton population. The FFQ was developed for use in the Southampton Women's Survey (SWS); this FFQ included questions on consumption frequency of distinct fish categories and reflected diet over the previous three months. The SWS FFQ has been validated for use to assess nutrient intakes in pregnant women in Southampton (214-219). Although weighed food diaries are considered to be more accurate when determining nutrient intake for a small number of volunteers, compared to FFQ, they cannot reflect a long period of time which was of interest in SIPS (103, 214). Also, prospective, open-ended dietary methods such as food diaries are demanding to fill in (12). Epidemiological studies investigating the effect of maternal fish intake during pregnancy on atopic outcomes in the offspring have also used FFQ as a method for determining intake of different types of fish (154-159).

The macronutrient intake calculated here for the SIPS subjects is very close to the that reported by SWS (219), showing that the FFQ was a reliable tool to estimate nutrient intake although the study group had a small size. Also, these similarities along with the fact that SIPS correlation coefficients from principal component analysis (PCA) correlated very well with the SWS coefficients shows that, apart from the SIPS subjects having a low intake of oily fish and being atopic, they were very similar to the average female Southampton population of this age and representative of that.

A limitation of the FFQ, as with most FFQ, is that it had an ordinal scale of distinct consumption frequency categories, rather than an interval scale providing a 'true' measure (220). As such, consumption of salmon twice per week was recorded as consumption 1-2 times per week, and was interpreted as 1.5 times per week for nutrient calculations. The fish diaries were important in determining a more precise consumption frequency. Also, volunteers filled in the diaries on a daily bases and so the recall bias associated with the FFQ was minimised. The use of fish diaries as a compliance measure was essential and can be considered as a strength of SIPS. The slight difference in the findings on consumption frequencies and nutrient intakes between FFQ and fish diaries may be again attributed to the limitation of the FFQ to identify small differences because of the distinct consumption frequency categories.

Also, a limitation of this specific FFQ is that it 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 containing supplements during pregnancy. Thus, it can be suggested that intake of EPA and DHA from total fish would be a very good estimate of total intake of these LC n-3 PUFA from the whole diet. The use of FFQ to assess micronutrient intake and especially LC n-3 PUFA has been validated in studies on pregnant (232, 233) and non-pregnant subjects (234, 235) and has also been reviewed (236). These studies support that the intake of EPA and DHA can be adequately estimated by FFQ.

The use of the FFQ allowed the investigation of other changes that may have occurred in the dietary pattern of the subjects associated with the intervention. However, a too long FFQ may be a limitation especially when conducting larger studies. The use of a shorter FFQ may be helpful when there is interest in only one aspect of the diet, such as vitamin D or fat (237), or fish intake and LC n-3 PUFA. Generally, a shorter FFQ, especially designed to determine consumption of food rich in the nutrients that are of interest can be less time consuming and equally efficient.

A disadvantage of the fish diaries is that they have not been validated. Also, fish diaries may have been subject to over or under reporting, as with any other dietary record method, especially when filled in for such a long period of time (12). Finally, the fish diaries were returned by a subset of subjects and not by the whole study group (77% in the control group, and 79% in the salmon group returned completed fish diaries).

There have been no studies of fish intervention during pregnancy. However the current study may be compared to other studies which investigated the effect of increased fish consumption on different health outcomes and on different populations (238-241, 243). Unlike the current study, these studies used food diaries as a method for measuring compliance and dietary intake.

The baseline difference in intakes of fish and nutrients from fish between the two SIPS groups, despite the fact that subjects were randomly assigned to the two arms, is a limitation of the current study. This was further reflected by the findings of the fatty acid analysis. At baseline (20 weeks gestation), the salmon group had significantly higher percentages of EPA, DHA, and total n-3 PUFA, and a lower percentage of arachidonic (ARA) in plasma phosholipids (PC) and in the other plasma lipid fractions, compared to the control group. Fasting plasma lipid fatty acids reflect fairly short-term changes in the

diet since they are associated, mainly, with continuous lipoprotein synthesis from the liver. However, cell fatty acid composition (especially red blood cells but also mononuclear cells) is affected in the long-term because of the long period of life of the cells (red blood cells have a half life of about 4 months). There were no baseline differences in the peripheral blood mononuclear cell (PBMC) fatty acid composition between the groups which may reflect that the salmon group consumed more fish closer to the start of the intervention (20 weeks) rather than habitually for a longer period.

The baseline differences in fish consumption and plasma LC PUFA levels between the two groups may be considered as a limitation of the current study. For this reason between group comparisons of the change in intake and in the fatty acid content between before and after starting the intervention were conducted. The increase in oily fish consumption, and in EPA and DHA intake, in the salmon group was significantly different from the consistently low intake in the control group (increase by 1.2 portions/week and by 269.3 mg/day, respectively).

Apart from increasing significantly EPA and DHA intake, the salmon intervention resulted in higher intake of vitamin D, selenium, and total n-3 PUFA from oily fish and total fish at 34 weeks gestation compared to the control group. More importantly, this increased intake from fish was reflected in an increased intake of these nutrients from total diet (estimated by FFQ). Total diet selenium intake was 58.2 μg/day for the salmon group (97% of Reference Nutrient Intake (RNI; 60 μg/day)) and 49 μg/day for the control group (82% RNI) (p = 0.005) (18). The contribution of study salmon to total diet intake of selenium was 20% which is quite substantial. The intake of selenium in the control group was fairly close to the average intake that has been recently reported by the National Diet and Nutrition Survey (NDNS) in the UK (39 μg/day) (13). It has to be noted that total diet selenium intake should be treated as a rough estimate, since the content of selenium in foods depends on soil content and data are not completely reliable (222). Total diet vitamin D intake was 5.75 μg/day for the salmon group (57% RNI; RNI for pregnant women is 10 μg/day) and 2.9 μg/day for the control group (29% RNI) (p < 0.001) (18). It is worth noting that, the study salmon contributed to 68% of the total vitamin D intake in the salmon group. Total diet vitamin D intake in the control group was very comparable to that reported by NDNS (2.7 μg/day) (13). These findings agree with the findings of other fish intervention trials (238, 243). In SIPS, it would be expected that the blood levels of selenium and vitamin D would be higher in the salmon group compared to the control group based on the fact that intake was significantly and substantially higher. Early fish and vitamin D exposure improves bone health in infancy and childhood (207, 208).

Maternal vitamin D intake during pregnancy has been associated with decreased risk of sensitization to food allergens in the offspring (209) and evidence shows the importance of vitamin D and selenium in reducing risk of atopic disease development (92, 210-213).

Consumption of two portions of oily fish per week would provide more than the minimum recommended intake of DHA and of EPA plus DHA (450 mg/day) (11), DHA (200 mg/day) (19) , 40% of the recommended intake of vitamin D for pregnant women (10 µg/day), and 20% of the recommended intake of selenium (60 µg/day). According to the findings of the fish diaries, consumption of two portions of study salmon per week (oily fish data including study salmon) provided 158.3 mg EPA, 322.2 mg DHA, 11.94 µg selenium, and 3.89 µg vitamin D per day. This would mean that consumption of two portions of oily fish per week would cover approximately 40% of the amount of vitamin D and selenium, 260% of the amount of EPA, 106% of the DHA, and 133% of total EPA plus DHA provided from a typical pregnancy supplement. This is the first study in pregnant women to provide this information, supporting the importance of oily fish during pregnancy as they can be an excellent source of a combination of nutrients. This, in relation to the fact that increased oily fish consumption resulted in lower meat intake, can provide strong evidence in order to support public health messages and interventions that target increased consumption of oily fish (at least two portions) instead of the use of fish oil supplements during pregnancy. Of course all considerations about limiting contaminant intake during pregnancy should be taken into account when choosing the source of fish.

The dietary intake of LC n-3 PUFA was reflected by the fatty acid analysis not only at baseline, as mentioned above, but also at 34 and 38 weeks during intervention. It has to be noted that fatty acid status in the current study was expressed in two different ways (percentage levels and absolute concentrations). This is a strength of the current study as there is no indication for any expression being more advantageous than the other. The combination of the two expressions gives a better picture of the changes that occur. Also, fatty acid analysis in the four plasma lipid fractions is another strength of the study which allowed for more specific changes to be observed.

The current study showed that there is an effect of pregnancy on fatty acid status of the mother, and this was differentiated from the effect of the salmon intervention. It was observed that pregnancy resulted in increased total plasma concentrations within each lipid fraction. The hyperlipidemia of pregnancy has been well established in literature (245, 255) and was seen in both groups in the current study without being affected by the salmon intervention. The increased concentration of lipids in maternal plasma during pregnancy,

especially of non-esterified fatty acids (NEFA) and triglycerides (TAG), is important for ensuring supply of fatty acids to the foetus via the placenta.

Specific pregnancy associated changes in fatty acid status were observed in the control group. In total plasma, ARA, EPA, DPA and DHA concentrations decreased during pregnancy. Changes were also observed within each lipid fraction. More specifically, ARA and LC n-3 PUFA concentrations decreased in plasma phospholipid (PC) whereas they increased in plasma NEFA, and either increased or remained stable in cholesteryl esters (CE) and TAG. On the other hand, expressed as percentages these fatty acids decreased in almost all lipid fractions. The current findings agree with existing literature that the absolute concentrations of ARA and DHA increase during pregnancy but that their percentages in various plasma lipids decrease (260, 261, 265, 270, 271). The agreement between fatty acid levels in the current study, which was conducted on a UK population of low intake of oily fish, with studies from other North Western European countries (Germany, the Netherlands, Belgium) may imply similar dietary patterns in these 'western-type' environments and may also be associated to the similarity of the ethnic groups. Also, these results suggest that the mother is progressively depleted in these fatty acids (especially DHA and ARA) during pregnancy as they are transported to the foetus to be used for foetal growth, visual and neuronal development (245, 251). The decrease in EPA is also associated to the fact that it is metabolised to DPA and eventually to DHA which is highly demanded by the foetus (47, 245, 251). The pregnancy associated LC n-3 PUFA and ARA depletion observed in the control group also reflects its very low intake of fish and thus of EPA and DHA. Low intake of these LC n-3 PUFA results in remarkable depletion of these fatty acids during pregnancy and may lead to alterations in the function of the maternal immune system and have implications on the provision of these fatty acids to the foetus, especially in multiparus women whose LC n-3 PUFA status has been progressively depleted throughout previous pregnancies (7, 47, 121).

The current study showed for the first time that consumption of two portions of salmon per week from week 20 of pregnancy until delivery reversed the pregnancy associated decrease in plasma EPA, DPA and DHA, resulting in an increase of these fatty acids in all plasma lipid fractions. In contrast, the salmon intervention enhanced the pregnancy associated ARA decrease in maternal plasma lipids. The ratios of fatty acids of interest (ARA/EPA, ARA/DHA, ARA/(EPA+DHA), and n-6/n-3) were lower in the salmon group compared to control at 34 and at 38 weeks gestation for all plasma lipid fractions. The data show that increased maternal intake of EPA and DHA through consumption of two portions of salmon per week increased their status in LC n-3 PUFA

which was compensated by a lower status of ARA. The lower ARA/DHA ratio in the salmon group may have an impact on the relative transfer of these fatty acids to the foetus via fatty acid transport proteins on the placenta for which these fatty acids may compete (189, 258). It can be speculated that the increased maternal status in EPA and DHA may have resulted in higher provision of these fatty acids to the foetus which may have beneficial implications for the foetal growth, visual and brain development, and also immune system development. In addition to that, the reversal of the decrease in LC n-3 PUFA may be associated to benefits for the mother herself and for her immune system.

Consumption of two portions of salmon per week during pregnancy resulted in higher EPA, DPA, and DHA content in maternal plasma NEFA. This reflects a higher adipose tissue storage of these fatty acids which was achieved by the salmon intervention. This is of high importance not only for foetal supply with LC n-3 PUFA during pregnancy but also for infant supply through breast milk after birth. It has been well established that fatty acids in breast milk originate from maternal adipose tissue fatty acids stored during pregnancy (160, 272). A higher supply of these fatty acids to the infant during the critical window of the initial post-partum period is also vital for its growth and development and potentially for its immune system modulation.

The fish intervention studies of Vidgren *et al*. (243), Pot *et al.* (241), and Din et al. (238), although not conducted on pregnant women, showed a higher level of EPA and DHA in fish consuming subjects and a lower level of ARA compared to control subjects. The current study adds to this body of literature as it is a novel study of fish intervention on pregnant women, showing that increased oily fish consumption can increase status of LC n-3 PUFA even during pregnancy when the maternal LC n-3 PUFA stores are normally depleted for the benefit of the foetus.

Zhang *et al.* (274) investigated maternal dietary intake of EPA and DHA and levels of these fatty acids in plasma PC of pregnant women and their neonates from river/lake, coastal, and inland regions of China. Dietary intake was assessed using a validated semi-quantitative FFQ. Median daily intake of EPA and DHA was higher in the river/lake and coastal regions compared to inland, probably due to higher intake of fish and other seafood. The median maternal plasma PC levels of DHA and EPA were higher in the river/lake and coastal regions compared to the inland region. These findings agree with the current study showing a close association between dietary intake of EPA and DHA and the corresponding levels of these fatty acids in maternal plasma PC. Also, De Vriese *et al.*

(266) showed that maternal plasma PC EPA and DHA percentages were positively associated with intake of these fatty acids during pregnancy.

A novel finding of the current study is that pregnancy had an effect on the fatty acid composition of immune cells. Maternal PBMC EPA, DPA, DHA and ARA content decreased during pregnancy. The pregnancy-associated decrease in PBMC LC n-3 PUFA and ARA suggests that immune cells 'give up' these fatty acids in order for them to be transferred to the foetus through the placenta. The current data support that these fatty acids essential for foetal growth and development are mobilised from the maternal cells to plasma lipoprotein CE and TAG fractions, and eventually plasma NEFA, that play a key role in transporting fatty acids to the foetus. Whether the change in fatty acid composition of PBMC is important in influencing (or regulating) pregnancy-associated changes in immune function is not clear, but this is an intriguing possibility since EPA, DHA and ARA all play a role in immune cell function.

It was shown that consumption of two portions of salmon per week not only prevented the pregnancy associated decrease in PBMC EPA and DHA status, but it also resulted in an increase of PBMC DHA. Moreover, the salmon intervention resulted in further decrease of maternal PBMC ARA content during pregnancy. The decrease of maternal immune cell content in EPA and DHA during pregnancy, and its reversal by the salmon intervention was considered to potentially have an impact on the function of the immune cells, including cytokine and eicosanoid production, which was further investigated and is discussed here later on.

The current findings on increased maternal LC n-3 PUFA status agree with the findings of fish oil supplementation studies. The study of Damsgaard *et al.* (275) showed that fish oil supplementation in healthy men resulted in higher levels of EPA and DHA, and lower levels of ARA in PBMC, as seen in the current study in the salmon group. Hawkes *et al.* (276) conducted a trial with DHA-rich tuna oil in healthy mothers from day 3 post-partum. After 4 weeks of supplementation, DHA concentrations in maternal plasma, PBMC, breast milk and breast milk cell PC increased in a linear manner in response to dietary DHA. In the high DHA intake group, percentages of PBMC PC EPA and DHA were significantly higher than those of the placebo group. This finding agrees with the current findings in the salmon group. In the study of Dunstan *et al*. (180) pregnant women were supplemented with a high dose of fish oil or placebo between week 20 gestation and delivery. At 30 and 37 weeks gestation, fish oil supplementation significantly increased maternal erythrocyte PC EPA and DHA percentages and significantly decreased the

percentages of all n-6 PUFA including ARA compared to baseline. The current study did not examine erythrocyte lipids. In the European multi-center study of Krauss-Etschmann *et al*. (184), pregnant women received a supplement providing 500 mg/day DHA plus 150 mg/day EPA or placebo from week 22 of gestation to delivery. Maternal plasma PC DHA and EPA levels increased from 20 weeks to 30 weeks and at delivery in the fish oil group. In the current study salmon increased plasma PC EPA and DHA compared with baseline and compared with control, although the increases seen were not as great as those of Krauss-Etschmann *et al*. (184). Krauss-Etschmann *et al*. (184) showed that the intervention also increased DHA levels in cord plasma PC. In the same study, Larque *et al.* (189) showed that fish oil supplementation increased the levels of DHA in placenta PC, whereas ARA levels remained unchanged. In the current study placenta DHA (and EPA) increased, but ARA declined.

Houwelingen *et al.* (264) found that maternal plasma PC fatty acids expressed as a percentage were highly significantly correlated with foetal plasma PC fatty acids. Vlaardingerbroek & Hornstra (265) concluded that maternal fatty acid percentages at delivery in red blood cell and plasma PC were highly correlated with neonatal fatty acid percentages at birth. Also, De Vriese *et al.* (266) showed that EPA and total n-6 PUFA levels in cord plasma PC were positively correlated with maternal dietary intake of these fatty acids during pregnancy. Zhang *et al.* (274) showed that neonates in inland regions of China (where fish consumption is lower) had higher levels of ARA and lower levels of EPA and DHA in their plasma PC compared to those in the lake/river and coastal regions and that maternal plasma EPA and DHA were also positively associated with the corresponding levels in cord plasma PC. Krauss-Etschmann *et al*. (184) showed that maternal fish oil supplementation in pregnancy increased DHA levels in cord plasma PC. These studies may support that the increased maternal LC n-3 PUFA status observed in the current study might be reflected also in the foetus benefiting its growth and development.

Placenta fatty acid content might reflect the nutrient supply to the foetus, and its analysis may be a good indicator of placental fatty acid transfer (247, 248). The increased provision of LC n-3 PUFA to the foetus in the current study is supported by the fact that consumption of two portions of salmon per week, between mid pregnancy to delivery, resulted in higher LC n-3 PUFA content in placenta and umbilical cord tissue. The placenta will have been built up using those fatty acids available from the maternal bloodstream and thus some relationship or similarity between placental and maternal plasma fatty acid compositions might be expected. Furthermore, in the current study percentages of ARA, EPA and DHA in total placental lipids in the control group were in

good agreement with studies in Germany and UK (16, 247). Placental PC from a Spanish population had higher proportions of EPA and DHA and a slightly lower proportion of ARA compared to the control group of the current study. These differences might reflect differences in maternal diet including differences in fish intake (273). In the study of Krauss-Etschmann *et al*. (184), Larque *et al.* (189) showed that fish oil supplementation during pregnancy increased the levels of DHA in placenta PC, whereas ARA levels remained unchanged. In the current study placenta DHA (and EPA) increased, but ARA declined.

Based on the findings discussed above, increased intake of salmon during pregnancy resulted in higher n-3 PUFA status in maternal plasma and immune cells. Thus, it was considered that salmon intake in pregnancy could influence maternal immunity that could impact on development of the foetal immune system and predisposition to atopy. Fatty acid-derived mediators might play a role in the immune changes that occur during pregnancy and in setting up the foetal immune system (93). The results obtained, indicate that, while there were some effects of salmon intake on certain immune parameters, the hypothesis that the fish intervention will alter maternal immunity cannot be accepted (i.e. increased salmon intake during pregnancy had only a limited impact on maternal immunity, at least as measured here). Furthermore the data obtained show that the effect of progression through pregnancy on immune function is of a much greater magnitude than the effect of salmon: a number of immune measures were significantly affected by progression through pregnancy.

Pregnancy is known to be associated with changes in maternal immune function, believed to be important to prevent rejection of the foetus which could be seen as an allograft (i.e. transplant) that would normally elicit an immune response resulting in its rejection (64, 65, 277). Thus the immune changes that occur allow a successful pregnancy to ensue. At birth several aspects of the foetal immune response are like those in the mother. Some foetal immune responses indicate a predisposition to atopy (291, 292).

The immune cell subset analysis showed that the percentage of B-cells in lymphocytes decreased during pregnancy, whereas the percentage of helper T-cells in total leukocytes (and their number in blood) increased during pregnancy. Also, in the current study it was shown that the number of cytotoxic T-cells in blood increased during pregnancy (but not their percentage).These findings are supported by other studies and are consistent with the immune shifts observed during normal pregnancy (64, 65, 315-317). Moreover, the percentage of T-regulatory cells in total leukocytes (and their number in blood) increased

during pregnancy. This finding is in accordance with previous literature findings on T-regulatory cell pool expansion (i.e. increase in number) during normal pregnancy (318-320). The percentage of toll-like receptor-2 positive (TLR-2^+) monocytes in total leukocytes, as well as their number, increased during pregnancy. This indicates an upregulation of innate immunity during pregnancy which is in agreement with current literature that the innate immune system is important during pregnancy (322-324).

In the current study the increase in helper T-cells (as a % of leukocytes) was greater in the salmon group compared to the control group. There appears to be no literature on fish or fish oil intake and maternal immune cell subsets in pregnancy with which to compare this finding. Since an increase in helper T-cells as a % of leukocytes is normally seen during pregnancy and this finding may indicate better progression to successful pregnancy with increased salmon intake.

Also it was found that the percentage of lymphocytes as natural killer (NK) cells (% lymphocytes) decreased in the salmon group but not in the control group. An increase in number and/or activity of NK cells and their subsets ($CD56^+/CD16^+$) has been observed in women with recurrent spontaneous abortions (RSA) (282). Also, it has been shown that the percentage of NK cells is lower in pregnant compared to non-pregnant women (315). This would suggest that a suppression in NK cell numbers and activity would promote successful pregnancy. Thus this effect of salmon may be an important and beneficial one. However, what happens in the maternal periphery may not necessarily reflect what happens in the materno-foetal interface. The only study available with which to compare these findings with NK cells in the salmon group is that of Krauss-Etschmann *et al.* (72) which showed that fish oil supplementation during pregnancy was associated with decreased frequencies of NK cells and $CCR3^+CD8^+$ T-cells in cord blood. However, that study did not investigate the effect of fish oil intervention on maternal immune cell subsets during pregnancy. There are no previous studies on fish oil supplementation or fish consumption during pregnancy that have reported results on maternal immune cell subsets.

One limitation of the current study is that intracellular markers that would characterise the functional properties of immune cell subsets and differentiate them into T-helper type 1 (Th1) or T-helper type 2 (Th2) phenotypes were not investigated. However, the current study did examine production of Th1 and Th2-type cytokines by unstimulated and stimulated mononuclear cells, a measure that does provide insight into the functional phenotype of the cells present. Also, it has to be noted that distinguishing between immunological changes that occur locally in the uterus and systemically in the maternal

circulation is important (322). Whether the salmon intervention affected frequencies and/or functionality of immune cell subsets in the materno-foetal interface is unknown and was not investigated here.

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, a strength was the method of cytokine determination. They were measured using a flow cytometry based assay termed cytometric bead array, which measures a range of cytokines characteristic for different responses: Th1 (interleukin (IL)-2, tumour necrosis factor (TNF)-α, interferon (IFN)-γ), Th2 (IL-4, IL-2) and regulatory (IL-10).

The production of all cytokines determined (IL-10, TNF-α, IFN-γ, IL-2, IL-4, IL-5) by maternal PBMC stimulated with mitogen (PHA) decreased during the course of pregnancy, as did the production of IL-10 in response to allergens. These observations suggest T cell reactivity decreases as pregnancy proceeds. Furthermore, the production of IFN-γ by TLR (-2, -3, -4) ligand stimulated PBMC decreased during pregnancy. The ratio of prototypical Th1 to Th2 cytokines (IFN-γ/IL-4) produced in response to PHA and the ratio of Th1 to T-regulatory cytokines (IFN-γ/IL-10) both decreased significantly during pregnancy. These changes signify a polarisation of the helper T-cells towards the Th2 and T-regulatory phenotype during pregnancy which is in agreement with current literature (277, 278, 329). Ho *et al.* (325) found that the proportion of IL-4 secreting cells exceeded IFN-γ secreting cells in peripheral blood and in decidua of normal pregnancies. Langer-Gould *et al.* (326) showed that IFN-γ producing $CD8^+$ T-cells decreased during the course of pregnancy. The findings of the current study are consistent with these studies. The findings of the current study also partly agree with those of Breckler *et al.* (287). It was shown that for both allergic and non-allergic women, although the regulatory responses (IL-10) did not change during pregnancy, Th1 responses (IFN-γ) were suppressed compared to postpartum. Non-allergic women downregulated Th2 responses (IL-13) during pregnancy, while allergic women failed to do so and continued to exhibit high Th2 responses. The authors suggested that these peripheral responses in allergic women may also skew the cytokine milieu at the maternal-foetal interface towards the Th2 phenotype, contributing to increased risk of atopy in the offspring of allergic mothers. This partly agrees with the current findings that Th1, Th2, and T-regulatory responses to PHA decreased during pregnancy in both groups.

Although results of the current study indicate that maternal immune responses during pregnancy were suppressed and polarised towards the Th2/T-regulatory phenotype, the

hypothesis that increased oily fish intake will alter cytokine expression by maternal PBMC during pregnancy was not supported. There were no major or consistent statistically significant differences between the two groups in terms of cytokine production. IL-10 production from salmon paralbumin stimulated PBMC decreased significantly in the control group whereas it remained stable in the salmon group. The same pattern was observed for IL-10 production in response to ovalbumin. This observation may be related to the increased salmon consumption of the intervention group which increased exposure of the subjects to salmon antigens. The levels of this regulatory cytokine (IL-10) may be pivotal for the regulation of the immune system of the foetus with increased exposure to salmon antigen through the materno-fetal unit, and may result in better tolerance of the offspring to salmon antigen (i.e. reduced likelihood of mounting an active immune response). Although the study of Breckler *et al*. (287) did not show any difference between during and after pregnancy for IL-10 production, other studies have shown that this cytokine is essential in regulating immune responses in pregnancy and that it is higher in the plasma of pregnant compared to non-pregnant women (330). The only study showing that fish oil supplementation affected maternal T-cell responses is the study of Krauss-Etschmann *et al.* (183). It was shown that fish oil supplementation during pregnancy was associated with lower mRNA expression of IL-1 and IFN-γ and higher mRNA expression of the regulatory cytokine transforming growth factor-β (TGF-β) in maternal blood at delivery. Thus, it appeared that fish oil supplementation during pregnancy downregulates Th1 responses in the mother (183). This agrees with the current observation that the maternal immune system was suppressed and polarised towards Th2/T-regulatory responses during pregnancy in both groups. Warstedt *et al.* (188) conducted a fish oil supplementation trial during pregnancy and lactation. No differences in secretion of chemokines and cytokines (IL-1β, TNF, IL-6, IL-10, IL-12, IFN-γ) were observed in the mothers with fish oil supplementation which agrees with the findings of the current study. In the current study, TNF-α production in response to TLR-2 ligand was not affected in the control group, but it increased and then it decreased in the salmon group. This may indicates a greater upregulation of innate immunity in the salmon group during pregnancy. Since innate immune system is important during pregnancy (322-324) this may indicate a better progression to successful pregnancy in the salmon group.

Both *in vitro* and animal feeding studies showed that LC n-3 PUFA inhibit T-cell proliferation and production of IL-2 and IFN-γ (126, 128). However, the results of human supplementation studies are heterogeneous (105, 112, 132, 331, 332). These studies, although not conducted on pregnant women, have shown an effect of increased EPA and

DHA intake on T-cell function which was not observed in the current study. The lack of effect in the current study may be due to the lower dose of EPA and DHA provided here.

Studies of maternal fish oil supplementation during pregnancy or lactation have shown effects on immune markers (including cytokine production in cord blood) in the offspring consistent with decreased risk of developing atopy (22, 178, 183, 185). Also, it has been shown that fish oil supplementation during pregnancy resulted in decreased risk of developing clinical signs of atopy in the offspring (186, 187). Moreover, there are various studies of fish oil supplementation in infancy or childhood showing that fish oil supplementation in early life may affect immune function in a protective way (196, 197, 199), although the evidence is inconsistent (103).

According to the above (22, 178, 183, 185-188, 196, 197, 199), although studies of maternal fish oil supplementation have not reported major changes on maternal T-cell function (apart from the study of Krauss-Etschmann *et al*.), they showed that early fish oil provision is associated with T-cell function changes in cord blood which may be consistent with decreased risk of developing atopy. Thus, the finding that maternal T-cell responses were not altered by the salmon intervention cannot exclude the possibility that the intervention might have affected foetal T-cell responses and subsequently immune system changes in the offspring. Also, the evidence available suggests that maternal fish oil supplementation may have a protective effect against the development of atopy clinical outcomes in the offspring early in life with a possible persistence until adolescence (103). It may be proposed that immune function changes observed in the offspring are not associated with immune function changes in the maternal blood periphery but in the decidua. Also, production of lipid mediators, such as eicosanoids, and the increased provision of LC n-3 PUFA from the study salmon through the placenta to the foetus may be more important in programming the immune system of the offspring.

As discussed earlier, changes in PBMC fatty acid composition were observed as an effect of pregnancy and also as an effect of the salmon intervention. Pregnancy was associated with PBMC ARA decrease in both groups. ARA from the cell membrane lipids is converted by COX to prostaglandin (PG) E_2. PGE_2 production from unstimulated and PHA-stimulated PBMC decreased during pregnancy. Thus the effect of pregnancy on PGE_2 production may simply relate to reduced ARA availability. In agreement with this, it was shown that PBMC ARA content correlated significantly and positively with PGE_2 production, whereas there was a significant and negative correlation between PBMC EPA and DHA, and PGE_2 production. Why PGE_2 production in response to lipopolysaccharide

(LPS; TLR-4 ligand) was not affected by pregnancy is not clear. There are no previous studies that have looked at PGE_2 production from cultured PBMC during pregnancy. At 34 weeks gestation PGE_2 expression by unstimulated mononuclear cells was lower in the salmon group. The pregnancy associated reduction in PGE_2 production by unstimulated PBMC occurred more quickly in the salmon group, probably associated with the lower PBMC ARA levels observed in the salmon group compared to the control group. One limitation in further interpreting these results is that other lipid mediators such as PGE_3, leukotriene (LT) B_4, LTB_5, resolvins, and protectins were not measured. The determination of other mediators would help in showing that higher EPA and DHA content in PBMC, due to salmon intake, leads to the production of less potent mediators.

In accordance to the current findings, it has been shown that fish oil supplementation results in decreased production of PGE_2 (112, 313). Warstedt *et al.* (188) conducted a fish oil supplementation trial during pregnancy (from week 25) and lactation. They showed a tendency towards decreased LPS-induced PGE_2 secretion from whole blood cultures in the fish oil group. No differences in secretion of LTB_4 were observed in the mothers with fish oil supplementation (188). These results partly agree with the current findings supporting the idea that increased intake of EPA and DHA may result in lower PGE_2 production in the maternal periphery but also in the materno-foetal interface resulting in a less inflammatory environment for the foetus, independently of cytokine production, and thus reduction of the risk of developing atopy in the offspring. The study of Prescott & Dunstan (182) 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 also in SIPS increased provision of EPA and DHA to the foetus will result in decreased production of ARA derived lipid mediators and increased production of the less potent EPA and DHA derived lipid mediators (PGE_3, LTB_5) from the offspring's immune cells. This may affect the offspring's T-cell function and polarisation and it may also predispose to a less inflammatory environment during early life, contributing to lower risk of developing atopy in the offspring related to increased maternal oily fish intake during pregnancy.

One limitation of the current study is the criterion used to determine pregnant women whose offspring were at high risk of developing atopy. This was based on self-reported clinical signs of atopy for the mother, the father, or sibling. Maternal and paternal atopy was also examined using skin prick testing (SPT), but only after the end of the intervention when this had been approved by the ethics committee. It was shown that the majority of the subjects who reported atopy also had positive SPT to at least one allergen, however it was not possible to examine all subjects since some of them had already dropped out. It

would have been more appropriate to perform the SPT at baseline and to include this to the selection criteria of the study. However, this was not possible because of constraints associated with obtaining ethics approval.

Finally, in order to explore whether the salmon intervention affected other parameters of the diet, the FFQ provided information on dietary patterns during and after the end of the intervention. These are discussed in the following paragraphs.

The PCA results described showed that the salmon group had a statistically significant higher 'prudent' diet z-score than the control group, indicating a 'healthier' dietary pattern for the salmon group, at baseline and at 34 weeks gestation. The higher 'prudent' diet scores in the salmon group, reflecting 'healthier' dietary patterns, are in accordance with the higher fish consumption both at baseline and at 34 weeks. It may be considered that volunteers in the salmon group made an effort to eat more healthily as they were aware of the fact that they were taking part in a nutrition intervention, and they belonged to the salmon group. Many volunteers reported verbally during appointments that they were trying to combine salmon with 'healthier' dietary choices, such as cooking it with vegetables, and generally taking care of their diet in total, as they realised that this would be good for themselves and the baby.

An interesting question to be addressed, through the dietary information available in SIPS, was whether the salmon intervention resulted in changes of dietary patterns during the intervention. The data showed that increased salmon consumption was compensated by a decreased consumption of other fish (non-oily fish and fish dishes according to fish diaries) and also by a decrease in red meat, chicken, and total meat intake. Another fish intervention did not observe any changes in meat intake, which is difficult to explain but may be related to that study using food diaries which do not reflect long term consumption (241, 242).

The information from the 3 month post-partum FFQ was used to investigate whether the intervention had an impact on fish consumption habits after its end. For the majority of the volunteers this increase was not sustained after the end of the intervention, as volunteers returned to their habitually low intake of oily fish. Interestingly, at 3 months post-partum 17% of the salmon group continued having oily fish more or equal to 1.5 times per week (i.e. $\geq$ 1-2 times per week) compared to 0% before intervention. This may suggest that, there was a sustained effect beyond the intervention period for only for a small number of volunteers. Similarly, the fish intervention study of Din *et al.* showed that

the nutrient intake differences observed during the study due to the intervention were not sustained after the end of the intervention (238). Borland *et al.* (215) examined the stability of dietary patterns in young women over a 2-year period. The study was performed on a subgroup of the SWS and it was concluded that dietary patterns were reasonably stable. This may explain the difficulty in changing dietary patterns and why high oily fish intake was not sustained after the end of SIPS intervention for the majority of the subjects in the salmon group. Investigating the factors that may help increase intake of oily and total fish by women of reproductive age in a sustainable way is an important issue that needs further exploration in order to be able to plan and implement successfully interventions that target increased fish intake.

In summary, SIPS is the first randomised control trial that successfully increased oily fish intake during pregnancy to the recommended level of intake (2 portions/week), showing that increased consumption of oily fish during pregnancy results in significantly increased intake of LC n-3 PUFA, vitamin D, and selenium. Consumption of two portions of oily fish per week provided more than the minimum recommended intake of DHA and of EPA plus DHA (109%), 60% of the recommended intake of vitamin D, and 20% of the recommended intake of selenium. The salmon intervention not only resulted in reversal of the maternal pregnancy-associated depletion in LC n-3 PUFA, but it also increased maternal status in LC n-3 PUFA, as measured in plasma lipids, immune cells and maternal tissues at birth. The implications of these results are that increased maternal EPA and DHA status may facilitate better transfer of these key fatty acids to the foetus which is vital for its growth and development. Although salmon intake resulted in increased maternal LC n-3 PUFA status this had only a limited impact on maternal immunity (at least as measured here). As a number of immune measures were significantly affected by progression through pregnancy, the effect of progression through pregnancy on immune function is of a much greater magnitude than the effect of salmon. Only a few immune markers were affected by the salmon intervention and these seem to indicate a better progression to successful pregnancy with increased salmon intake. Also, the salmon intervention resulted in different pattern of maternal regulatory cytokine (IL-10) responses to salmon and egg allergen which may be pivotal for the regulation of the immune system of the foetus with increased exposure to salmon antigen through the materno-fetal unit, and may result in better tolerance of the offspring to salmon antigen (i.e. reduced likelihood of mounting an active immune response). Immune effects at the materno-foetal interface which may have an important role in immune system development of the foetus were not investigated, and the possibility that the salmon intervention may have resulted in local immune alterations

cannot be excluded. The salmon intervention may have resulted in programming of the immune system of the foetus and the developing offspring, mediated, not through changes in maternal immune cell phenotype and function, but through the provision of higher amounts of LC n-3 PUFA and lipid mediator alterations in the decidua. It was shown that there was a tendency for lower inflammatory eicosanoid production (PGE_2) in the salmon group which was associated with the lower ARA content in PBMC. This may be accompanied by increased eicosanoid and lipid mediators from EPA and DHA which have less inflammatory potency. This may potentially affect the offspring's T-cell function and polarisation and it may also predispose to a less inflammatory environment during early life, contributing to lower risk of developing atopy in the offspring related to increased maternal oily fish intake during pregnancy.

In conclusion, if pregnant women who do not habitually consume oily fish, eat two portions of oily fish (salmon) per week, from week 20 gestation to delivery, they will significantly increase their intake of LC n-3 PUFA (achieving recommended intake), vitamin D, and selenium, and they will also increase their status in LC n-3 PUFA. This may suggest increased availability of these fatty acids to the foetus which would facilitate its growth and development. The salmon intervention had only limited impact on maternal immunity as measured here, and thus it cannot be concluded whether the intervention would have an effect on the immune system of the offspring. Further investigation is needed on the mechanisms through which increased maternal fish consumption during pregnancy may affect the developing foetal immune system in a protective way against atopic disease development in the offspring later on in life.

6.1 Future work

The Salmon in Pregnancy Study (SIPS) design and findings presented in this thesis add valuable and novel information to the existing literature and encourage further future work on this area to be conducted. The present thesis focused on the effects of the intervention on maternal outcomes, although the main outcome measure of SIPS was the clinical signs of atopic disease manifestation in the offspring. Clinical follow-up of the offspring at 6 months of age has been completed. Findings from cord blood and from the 6 month clinical follow-up will be reported by Maria Vlachava in her PhD thesis. It was shown that the salmon intervention increased offspring long chain (LC) n-3 polyunsaturated fatty acid (PUFA) status at birth (i.e. in cord blood). Pro-inflammatory prostaglandin (PG) E_2 production by cord blood mononuclear cells was lower in the salmon group indicating exposure to a less inflammatory environment early in life which could be related to lower risk of developing atopy. Differences between the two groups were also observed in cytokine production from cord blood mononuclear cells which could be consistent with a less atopy-prone phenotype. However, at 6 months of age, clinical examination of the infants (skin prick testing (SPT) and eczema score) did not show any effects of the salmon intervention. Future follow-up of this cohort is essential as clinical manifestations of atopy may be more pronounced later on in life and thus differences in atopic disease development between the groups may be more evident as the children become older. A possible time point would be when the infants are 2.5 years of age (summer 2010-summer 2011). Apart from clinical signs on atopy, it would be of great interest to assess cognitive and visual development of the children. Although SIPS was not designed to assess these outcomes, it would be worth doing since there are no other randomised controlled trials (RCT) with fish during pregnancy. Subject to the findings at the 2.5 years of age follow-up, a next follow-up when children are 5 years old would be appropriate.

As mentioned repeatedly in this thesis, SIPS is the first fish intervention during pregnancy and this is partly related to the difficulties and practical implications of such an intervention. A larger RCT of increased oily fish consumption during pregnancy would be more adequately powered to detect differences in atopic outcomes, but also outcomes related to development such as length of gestation, cognition, and vision. An appropriate follow-up would be when the offspring are 6 months of age and later on at 1, 2.5, and 5 years. This would provide clearer evidence in support, or not, of the epidemiological studies which show a protective effect of fish consumption during pregnancy on atopic disease development. Such a study would require follow-up of the subjects' diet similar to the present study (a shorter and more focused food frequency questionnaire would be

equally effective) in order to prove compliance. However, maternal blood collection would not be necessary, making the study more easy to conduct, since it was shown by the present study that increased oily fish consumption (2 portions/week) increases maternal LC n-3 PUFA status (and had no major effect on maternal immunity). This would enable completion and increase feasibility of a larger RCT with fish during pregnancy. As it is vital to determine the mechanisms through which increased early exposure to nutrients from oily fish could be protective against atopic disease development, it may be worth investigating immunological changes that happen in the materno-foetal interface (e.g. immune markers in placenta, cytokines produced by immune cells in the placenta) rather than the maternal periphery, as this may show predisposition of the foetus to a less inflammatory environment as a result of increased maternal oily fish consumption. Examining the hypothesis that increased oily fish intake during pregnancy alters the foetal immune system during gestation is very difficult in humans. However, analysis of immune markers in cord blood at birth may provide an indication of changes in foetal immunity during gestation. Possible variations in the methodology and design of such a larger study in order to clarify the effect of increased oily fish during pregnancy would include: different levels of fish consumption (portions/week), different types of fish (including types of oily and non-oily fish), different early life critical windows (duration of intervention and period of gestation), different levels of baseline exposure to fish (never or <1/month, 1-2 times/month, 2/month). Also, determining the study population characteristics in relation to atopy risk at baseline more strictly than in SIPS would be an advantage. An example would be to include only pregnant women who had self-reported atopy and had a positive SPT to at least one allergen. It is obvious that a safe source of fish low in contaminants should be used. Such a study would require a significant amount of time and effort and would demand strong support and funding. Finally, within such a study or in a separate one, investigating the factors that may help to increase intake of oily and total fish by women of reproductive age in a sustainable way is important and needs exploration in order to be able to plan and implement successful interventions that target increased fish intake in this sub-group of the population.

References

1. Calder, P. C. (1998) Dietary fatty acids and lymphocyte functions. Proceedings of the Nutrition Society 57: 487-502.

2. Calder, P. C. (2001) Omega-3 polyunsaturated fatty acids, inflammation and immunity: Pouring oil on troubled waters or another fishy tale? Nutrition Research 21: 309-341.

3. Calder, P. C. (2005) Polyunsaturated fatty acids and inflammation. Biochemical Society Transactions 33: 423-427.

4. Sprecher, H. (1999) An update on the pathways of polyunsaturated fatty acid metabolism. Current Opinion in Clinical Nutrition & Metabolic Care 2: 135-138.

5. Calder, P. C. (2003) Omega-3 polyunsaturated fatty acids and inflammation: From molecular biology to the clinic. Lipids 38: 343-352.

6. Childs, C. E., Romeu-Nadal, M., Burdge, G. C. & Calder, P. C. (2008) Gender differences in the n-3 fatty acid content of tissues. Proceedings of the Nutrition Society 67: 19-27.

7. Calder, P. C. (2006) Omega-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. American Journal of Clinical Nutrition 83: S1505-S1519.

8. FSA & Food Standards Agency (2002) McCance and Widdowson's The composition of foods, sixth summary edition. Royal Society of Chemistry, Cambridge.

9. MAFF & Ministry of agriculture fisheries and food (1998) Fatty acids, seventh supplement to fifth edition of McCance and Widdowson's The composition of foods. Royal Society of Chemistry, Cambridge.

10. MAFF & Ministry of agriculture fisheries and food (1993) Fish and fish products, Supplement to McCance and Widdowson's The composition of Foods. Royal Society of Chemistry, Cambridge.

11. SACN, Scientific Advisory Committee on Nutrition & Committee on Toxicity (2004) Advice on fish consumption: benefits and risks. The Stationary Office, London.

12. Department of Health & Food Standards Agency (2004) National Diet and Nutrition Survey: Adults aged 19 to 64 years. The Stationary Office, London.

13. Department of Health & Food Standards Agency (2009) National Diet and Nutrition Survey: NDNS Headline results from Year 1 of the Rolling programme (2008/2009). The Stationary Office, London.

14. Givens, D. I. & Gibbs, R. A. (2006) Very long chain n-3 polyunsaturated fatty acids in the food chain in the UK and the potential of animal-derived foods to increase intake. Nutrition Bulletin 31: 104-110.

15. Meyer, B. J., Mann, N. J., Lewis, J. L., Milligan, G. C., Sinclair, A. J. & Howe, P. R. C. (2003) Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. Lipids 38: 398.

16. Lakin, V., Haggarty, P., Abramovich, D. R., Ashton, J., Moffat, C. F., McNeill, G., Danielian, P. J. & Grubb, D. (1998) Dietary intake and tissue concentration of fatty acids in omnivore, vegetarian and diabetic pregnancy. Prostaglandins Leukotrienes and Essential Fatty Acids 59: 209-220.

17. Masson, L. F., McNeill, G., Tomany, J. O., Simpson, J. A., Peace, H. S., Wei, L., Grubb, D. A. & Bolton-Smith, C. (2003) Statistical approaches for assessing the relative validity of a food-frequency questionnaire: Use of correlation coefficients and the kappa statistic. Public Health Nutrition 6: 313-321.

18. Department of Health (1991) Dietary Reference Values for food, energy, and nutrients for the United Kingdom. The Stationary Office, London.

19. Cetin, I. & Koletzko, B. (2008) Long-chain omega-3 fatty acid supply in pregnancy and lactation. Current Opinion in Clinical Nutrition & Metabolic Care 11: 297-302.

20. Simopoulos, A. P., Leaf, A. & Salem, N. J. (1999) Workshop on the essentiality of and Recommended Dietary Intakes for omega-6 and omega-3 fatty acids. Journal of the American College of Nutrition 18: 487-489.

21. Koletzko, B., Cetin, I. & Brenna, J. T. (2007) Dietary fat intakes for pregnant and lactating women. International Society for the Study of Fatty Acids and Lipids Newsletter 14: 12-17.

22. Dunstan, J. A., Mori, T. A., Barden, A., Beilin, L. J., Taylor, A. L., Holt, P. G. & Prescott, S. L. (2003) Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: A randomized, controlled trial. Journal of Allergy and Clinical Immunology 112: 1178-1184.

23. Al, M. D. M., van Houwelingen, A. C. & Hornstra, G. (2000) Long-chain polyunsaturated fatty acids, pregnancy, and pregnancy outcome. American Journal of Clinical Nutrition 71: 285S-291S.

24. Dunstan, J. A., Simmer, K., Dixon, G. & Prescott, S. L. (2008) Cognitive assessment of children at age 2 1/2 years after maternal fish oil supplementation in pregnancy: A randomised controlled trial. Archives of Disease in Childhood: Fetal and Neonatal Edition 93: F45-F50.

25. Oken, E., Kleinman, K. P., Olsen, S. F., Rich-Edwards, J. W. & Gillman, M. W. (2004) Associations of seafood and elongated n-3 fatty acid intake with fetal growth and length of gestation: results from a US pregnancy cohort. American Journal of Epidemiology 160: 774-783.

26. Olsen, S. F., Hansen, H. S., Sorensen, T. I., Jensen, B., Secher, N. J., Sommer, S. & Knudsen, L. B. (1986) Intake of marine fat, rich in (n-3)-polyunsaturated fatty acids, may increase birthweight by prolonging gestation. Lancet 2: 367-369.

27. Roitt, I., Brostoff, J. & Male, D. (2001) Immunology. 6th Edition, Harcourt Publishers Limited.

28. Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. (2005) Immunobiology. 6th Edition, London: Garland Publishing.

29. Chaplin, D. D. (2006) 1. Overview of the human immune response. Journal of Allergy & Clinical Immunology 117: S430-S435.

30. Yokoyama, W. M. & Colonna, M. (1920) Innate immunity to pathogens. Current Opinion in Immunology 1-2.

31. Liew, F. Y. & McInnes, I. B. (2002) The role of innate mediators in inflammatory response. Molecular Immunology 38: 887-890.

32. Woodland, D. L. (1920) Immunologic memory. Viral Immunology 229-230.

33. Alam, R. & Gorska, M. (2003) 3. Lymphocytes. Journal of Allergy & Clinical Immunology 111: S476-S485.

34. Grimble, R. F. (2005) Immunonutrition. Current Opinion in Gastroenterology 21: 216-222.

35. Woodland, D. L. & Dutton, R. W. (2003) Heterogeneity of CD4(+) and CD8(+) T cells. Current Opinion in Immunology 15: 336-342.

36. Maloy, K. J., Salaun, L., Cahill, R., Dougan, G., Saunders, N. J. & Powrie, F. (197) CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. Journal of Experimental Medicine 111-119.

37. Maloy, K. J. & Powrie, F. (2001) Regulatory T cells in the control of immune pathology. Nature Immunology 2: 816-822.

38. Calder, P. C. (2001) Polyunsaturated fatty acids, inflammation, and immunity. Lipids 36: 1007-1024.

39. Grimble, R. F. (2001) Nutritional modulation of immune function. Proceedings of the Nutrition Society 60: 389-397.

40. Rottem, M., Gershwin, M. E. & Shoenfeld, Y. (2002) Allergic disease and autoimmune effectors pathways. Developmental Immunology 9: 161-167.

41. Oboki, K., Ohno, T., Saito, H. & Nakae, S. (2008) Th17 and Allergy. Allergology International 57: 121-134.

42. Mosmann, T. R., Sad, S., Krishnan, L., Wegmann, T. G., Guilbert, L. J. & Belosevic, M. (1995) Differentiation of subsets of CD4+ and CD8+ T cells. Ciba Foundation Symposium. 195: 42-50.

43. Mosmann, T. R. & Sad, S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology Today. 17: 138-146.

44. Liew, F. Y. (2002) T(H)1 and T(H)2 cells: a historical perspective. Nature Reviews. Immunology 2: 55-60.

45. Sad, S. & Mosmann, T. R. (1994) Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. Journal of Immunology 153: 3514-3522.

46. Roark, C. L., Simonian, P. L., Fontenot, A. P., Born, W. K. & O'Brien, R. L. (2008) Gamma/Delta T cells: an important source of IL-17. Current Opinion in Immunology 20: 1-5.

47. Calder, P. C., Krauss-Etschmann, S., de Jong, E. C., Dupont, C., Frick, J.-S., Frokiaer, H., Heinrich, J., Garn, H., Koletzko, S. et al. (2006) Early nutrition and immunity - Progress and perspectives. British Journal of Nutrition 96: 774-790.

48. Rottem, M. & Shoenfeld, Y. (2003) Asthma as a paradigm for autoimmune disease. International Archives of Allergy & Immunology 132: 210-214.

49. Mathias, R. A., Freidhoff, L. R., Blumenthal, M. N., Meyers, D. A., Lester, L., King, R., Xu, J. F., Solway, J., Barnes, K. C. et al. (2001) Genome-wide linkage analyses of total serum IgE using variance components analysis in asthmatic families. Genetic Epidemiology 20: 340-355.

50. Sicherer, S. H., Furlong, T. J., Maes, H. H., Desnick, R. J., Sampson, H. A. & Gelb, B. D. (2000) Genetics of peanut allergy: a twin study. Journal of Allergy & Clinical Immunology 106: 53-56.

51. Strachan, D. P., Wong, H. J. & Spector, T. D. (2001) Concordance and interrelationship of atopic diseases and markers of allergic sensitization among adult female twins. Journal of Allergy & Clinical Immunology 108: 901-907.

52. Willemsen, G., Van Beijsterveldt, T. C. E. M., Van Baal, C. G. C. M., Postma, D. & Boomsma, D. I. (2008) Heritability of self-reported asthma and allergy: a study in adult Dutch twins, siblings and parents. Twin Research and Human Genetics 11: 132-142.

53. Yang, K. D., Ou, C. Y., Hsu, T. Y., Chang, J. C., Chuang, H., Liu, C. A., Liang, H. M., Kuo, H. C., Chen, R. F. & Huang, E. Y. (2007) Interaction of maternal atopy, CTLA-4 gene polymorphism and gender on antenatal immunoglobulin E production. Clinical & Experimental Allergy 37: 680-687.

54. Christensen, U., Haagerup, A., Binderup, H. G., Vestbo, J., Kruse, T. A. & Borglum, A. D. (2006) Family based association analysis of the IL2 and IL15 genes in allergic disorders. European Journal of Human Genetics 14: 227-235.

55. Donfack, J., Schneider, D. H., Tan, Z., Kurz, T., Dubchak, I., Frazer, K. A. & Ober, C. (2005) Variation in conserved non-coding sequences on chromosome 5q and susceptibility to asthma and atopy. Respiratory Research 6: 145.

56. Adjers, K., Karjalainen, J., Pessi, T., Eklund, C. & Hurme, M. (2005) Epistatic effect of TLR4 and IL4 genes on the risk of asthma in females. International Archives of Allergy & Immunology 138: 251-256.

57. Holloway, J. W. & Holgate, S. T. (2004) Genetics. Chemical Immunology and Allergy 84: 1-35.

58. Kurukulaaratchy, R. J., Matthews, S. & Arshad, S. H. (2004) Does environment mediate earlier onset of the persistent childhood asthma phenotype? Pediatrics 113: 345-350.

59. Liang, P. H., Shyur, S. D., Huang, L. H., Wen, D. C., Chiang, Y. C., Lin, M. T. & Yang, H. C. (2006) Risk factors and characteristics of early-onset asthma in Taiwanese children. Journal of Microbiology, Immunology & Infection 39: 414-421.

60. Cantani, A. & Micera, M. (2004) Natural history of cow's milk allergy. An eight-year follow-up study in 115 atopic children. European Review for Medical & Pharmacological Sciences 8: 153-164.

61. Sandin, A., Bjorksten, B. & Braback, L. (2004) Development of atopy and wheezing symptoms in relation to heredity and early pet keeping in a Swedish birth cohort. Pediatric Allergy & Immunology 15: 316-322.

62. Kokkonen, J. & Niinimaki, A. (2004) Increased incidence of autoimmune disorders as a late complication in children with early onset dermatitis and/or milk allergy. Journal of Autoimmunity 22: 341-344.

63. Warner, J. A. & Warner, J. O. (2000) Early life events in allergic sensitisation. British Medical Bulletin 56: 883-893.

64. Warner, J. O. (2007) Early life nutrition and allergy. Early Human Development 83: 777-783.

65. Raghupathy, R. (2001) Pregnancy: Success and failure within the Th1/Th2/Th3 paradigm. Seminars in Immunology 13: 219-227.

66. Prescott, S. L., Macaubas, C., Smallacombe, T., Holt, B. J., Sly, P. D. & Holt, P. G. (1999) Development of allergen-specific T-cell memory in atopic and normal children. Lancet 353: 196-200.

67. Jones, C. A., Vance, G. H. S., Power, L. L., Pender, S. L. F., MacDonald, T. T. & Warner, J. O. (2001) Costimulatory molecules in the developing human gastrointestinal tract: A pathway for fetal allergen priming. Journal of Allergy and Clinical Immunology 108: 235-241.

68. Thornton, C. A., Holloway, J. A., Popplewell, E. J., Shute, J. K., Boughton, J. & Warner, J. O. (2003) Fetal exposure to intact immunoglobulin E occurs via the gastrointestinal tract. Clinical & Experimental Allergy 33: 306-311.

69. Jones, C. A., Miles, E. A., Warner, J. O., Colwell, B. M., Bryant, T. N. & Warner, J. A. (1996) Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. Pediatric Allergy and Immunology 7: 109-116.

70. Vance, G. H. S., Grimshaw, K. E. C., Briggs, R., Lewis, S. A., Mullee, M. A., Thornton, C. A. & Warner, J. O. (2004) Serum ovalbumin-specific immunoglobulin G responses during pregnancy reflect maternal intake of dietary egg and relate to the development of allergy in early infancy. Clinical and Experimental Allergy 34: 1855-1861.

71. Upton, M. N., McConnachie, A., McSharry, C., Hart, C. L., Smith, G. D., Gillis, C. R. & Watt, G. C. M. (2000) Intergenerational 20 year trends in the prevalence of asthma and hay fever in adults: The Midspan family study surveys of parents and offspring. British Medical Journal 321: 88-92.

72. Woolcock, A. J. & Peat, J. K. (2006) Evidence for the increase in asthma worldwide. Ciba Foundation Symposium. 206: 122-134.

73. Aberg, N., Hesselmar, B., Aberg, B. & Eriksson, B. (1995) Increase of asthma, allergic rhinitis and eczema in Swedish schoolchildren between 1979 and 1991. Clinical and Experimental Allergy 25: 815-819.

74. Ninan, T. K. & Russell, G. (1992) Respiratory symptoms and atopy in Aberdeen schoolchildren: Evidence from two surveys 25 years apart. British Medical Journal 873-875.

75. Leibowitz, U., Antonovsky, A., Medalie, J. M., Smith, H. A., Halpern, L. & Alter, M. (1966) Epidemiological study of multiple sclerosis in Israel. II. Multiple sclerosis and level of sanitation. Journal of Neurology, Neurosurgery & Psychiatry 29: 60-68.

76. Strachan, D. P. (1989) Hay fever, hygiene, and household size. British Medical Journal 299: 1259-1260.

77. Bach, J. F. (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. New England Journal of Medicine 347: 911-920.

78. Bach, J. F. (2005) Infections and autoimmune diseases. Journal of Autoimmunity 25: S74-S80.

79. Black, P. N. & Sharpe, S. (1997) Dietary fat and asthma: is there a connection? European Respiratory Journal 10: 6-12.

80. Hodge, L., Peat, J. K. & Salome, C. (1994) Increased consumption of polyunsaturated oils may be a cause of increased prevalence of childhood asthma. Australian & New Zealand Journal of Medicine 24: 727.

81. Tricon, S., Willers, S., Smit, H. A., Burney, P. G., Devereux, G., Frew, A. J., Halken, S., Host, A., Nelson, M. et al. (2006) Nutrition and allergic disease. Clinical and Experimental Allergy Reviews 6: 117-188.

82. Duchen, K., Yu, G. & Bjorksten, B. (1998) Atopic sensitization during the first year of life in relation to long chain polyunsaturated fatty acid levels in human milk. Pediatric Research 44: 478-484.

83. Kankaanpaa, P., Nurmela, K., Erkkila, A., Kalliomaki, M., Holmberg-Marttila, D., Salminen, S. & Isolauri, E. (2001) Polyunsaturated fatty acids in maternal diet, breast milk, and serum lipid fatty acids of infants in relation to atopy. Allergy: European Journal of Allergy and Clinical Immunology 56: 633-638.

84. Oddy, W. H., Pal, S., Kusel, M. M. H., Vine, D., De Klerk, N. H., Hartmann, P., Holt, P. G., Sly, P. D., Burton, P. R. et al. (2006) Atopy, eczema and breast milk fatty acids in a high-risk cohort of children followed from birth to 5 years. Pediatric Allergy and Immunology 17: 4-10.

85. Stoney, R. M., Woods, R. K., Hosking, C. S., Hill, D. J., Abramson, M. J. & Thien, F. C. K. (2004) Maternal breast milk long-chain omega-3 fatty acids are associated with increased risk of atopy in breastfed infants. Clinical and Experimental Allergy 34: 194-200.

86. Thijs, C., Van, H. A., Poorterman, I., Mordant, A. & Van Den, B. P. (2000) Essential fatty acids in breast milk of atopic mothers: Comparison with non-atopic mothers, and effect of borage oil supplementation. European Journal of Clinical Nutrition 54: 234-238.

87. Calder, P. C. (2009) Early life programming of immune and lung function: can we now eclude a role of arachidonic acid exposure? British Journal of Nutrition 102: 331-333.

88. Calder, P. C. (2006) Abnormal fatty acid profiles occur in atopic dermatitis but what do they mean? Clinical & Experimental Allergy 36: 138-141.

89. Byberg, K., Oymar, K. & Aksnes, L. (2008) Fatty acids in cord blood plasma, the relation to soluble CD23 and subsequent atopy. Prostaglandins Leukotrienes and Essential Fatty Acids 78: 61-65.

90. Gold, D. R., Willwerth, B. M., Tantisira, K. G., Finn, P. W., Schaub, B., Perkins, D. L., Tzianabos, A., Ly, N. P., Schroeter, C. et al. (2006) Associations of cord blood fatty acids with lymphocyte proliferation, IL-13, and IFN-gamma. Journal of Allergy and Clinical Immunology 117: 931-938.

91. Yu, G., Kjellman, N.-I. & Bjorksten, B. (1996) Phospholipid fatty acids in cord blood: Family history and development of allergy. Acta Paediatrica, International Journal of Paediatrics 85: 679-683.

92. Shaheen, S. O., Newson, R. B., Henderson, A. J., Emmett, P. M., Sherriff, A. & Cooke, M. (2004) Umbilical cord trace elements and minerals and risk of early childhood wheezing and eczema. European Respiratory Journal 24: 292-297.

93. Prescott, S. L. & Dunstan, J. A. (2007) Prenatal fatty acid status and immune development: The pathways and the evidence. Lipids 42: 801-810.

94. Calder, P. C. (2007) Immunomodulation by omega-3 fatty acids. Prostaglandins Leukotrienes and Essential Fatty Acids 77: 327-335.

95. Serhan, C. N. (2005) Novel eicosanoid and docosanoid mediators: resolvins, docosatrienes, and neuroprotectins. Current Opinion in Clinical Nutrition & Metabolic Care 8: 115-121.

96. Lewis, R. A., Austen, K. F. & Soberman, R. J. (1990) Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. New England Journal of Medicine 323: 645-655.

97. Tilley, S. L., Coffman, T. M. & Koller, B. H. (2001) Mixed messages: Modulation of inflammation and immune responses by prostaglandins and thromboxanes. Journal of Clinical Investigation 108: 15-23.

98. Bagga, D., Wang, L., Farias-Eisner, R., Glaspy, J. A. & Reddy, S. T. (2003) Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on cyclooxygenase-2 expression and interleukin-6 secretion. Proceedings of the National Academy of Sciences of the United States of America 100: 1751-1756.

99. Levy, B. D., Clish, C. B., Schmidt, B., Gronert, K. & Serhan, C. N. (2001) Lipid mediator class switching during acute inflammation: Signals in resolution. Nature Immunology 2: 612-619.

100. Vachier, I., Chanez, P., Bonnans, C., Godard, P., Bousquet, J. & Chavis, C. (2002) Endogenous anti-inflammatory mediators from arachidonate in human neutrophils. Biochemical and Biophysical Research Communications 290: 219-224.

101. Gewirtz, A. T., Collier-Hyams, L. S., Young, A. N., Kucharzik, T., Guilford, W. J., Parkinson, J. F., Williams, I. R., Neish, A. S. & Madara, J. L. (2002) Lipoxin A4 analogs attenuate induction of intestinal epithelial proinflammatory gene expression and reduce the severity of dextran sodium sulfate-induced colitis. Journal of Immunology 168: 5260-5267.

102. Serhan, C. N., Jain, A., Marleau, S., Clish, C., Kantarci, A., Behbehani, B., Colgan, S. P., Stahl, G. L., Merched, A. et al. (2003) Reduced inflammation and tissue damage in transgenic rabbits overexpressing 15-lipoxygenase and endogenous anti-inflammatory lipid mediators. Journal of Immunology 171: 6856-6865.

103. Kremmyda, L. S., Vlachava, M., Noakes, P. S., Diaper, N. D., Miles, E. A. & Calder, P. C. (2009) 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 Online Publication DOI 10.1007/s12016-009-8186-2.

104. Peterson, L. D., Jeffery, N. M., Thies, F., Sanderson, P., Newsholme, E. A. & Calder, P. C. (1998) Eicosapentaenoic and docosahexaenoic acids alter rat spleen leukocyte fatty acid composition and prostaglandin E2 production but have different effects on lymphocyte functions and cell-mediated immunity. Lipids 33: 171-180.

105. Thies, F., Nebe-von-Caron, G., Powell, J. R., Yaqoob, P., Newsholme, E. A. & Calder, P. C. (2001) Dietary supplementation with gamma-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans. Journal of Nutrition 131: 1918-1927.

106. Yaqoob, P., Pala, H. S., Cortina-Borja, M., Newsholme, E. A. & Calder, P. C. (2000) Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. European Journal of Clinical Investigation 30: 260-274.

107. Kelley, D. S., Taylor, P. C., Nelson, G. J. & Mackey, B. E. (1998) Arachidonic acid supplementation enhances synthesis of eicosanoids without suppressing immune functions in young healthy men. Lipids 33: 125-130.

108. Thies, F., Miles, E. A., Nebe-von-Caron, G., Powell, J. R., Hurst, T. L., Newsholme, E. A. & Calder, P. C. (2001) Influence of dietary supplementation with long-chain omega-3 or omega-6

polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. Lipids 36: 1183-1193.

109. Lee, T. H., Hoover, R. L. & Williams, J. D. (1985) Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. New England Journal of Medicine 312: 1217-1224.

110. Healy, D. A., Wallace, F. A., Miles, E. A., Calder, P. C. & Newsholme, P. (2000) Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. Lipids 35: 763-768.

111. Meydani, S. N., Endres, S., Woods, M. M., Goldin, B. R., Soo, C., Morrill-Labrode, A., Dinarello, C. A. & Gorbach, S. L. (1991) Oral (omega-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: Comparison between young and older women. Journal of Nutrition 121: 547-555.

112. Trebble, T. M., Wootton, S. A., Miles, E. A., Mullee, M., Arden, N. K., Ballinger, A. B., Stroud, M. A., Burdge, G. C. & Calder, P. C. (2003) Prostaglandin E2 production and T cell function after fish-oil supplementation: response to antioxidant cosupplementation. American Journal of Clinical Nutrition 78: 376-382.

113. Caughey, G. E., Mantzioris, E., Gibson, R. A., Cleland, L. G. & James, M. J. (1996) The effect on human tumor necrosis factor alpha and interleukin-1beta production of diets enriched in omega-3 fatty acids from vegetable oil or fish oil. American Journal of Clinical Nutrition 63: 116-122.

114. Sperling, R. I., Benincaso, A. I., Knoell, C. T., Larkin, J. K., Austen, K. F. & Robinson, D. R. (1993) Dietary omega-3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. Journal of Clinical Investigation 91: 651-660.

115. Von, S., Kiefl, R., Jendraschak, E. & Kaminski, W. E. (1993) Omega-3 fatty acids and cysteinyl-leukotriene formation in humans in vitro, ex vivo, and in vivo. Journal of Laboratory and Clinical Medicine 121: 302-309.

116. Hawkes, J. S., James, M. J. & Cleland, L. G. (1991) Separation and quantification of PGE3 following derivatization with panacyl bromide by high pressure liquid chromatography with fluorometric detection. Prostaglandins 191: 355-368.

117. Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Gronert, K. & Chiang, N. (2000) Anti-microinflammatory lipid signals generated from dietary omega-3 fatty acids via cyclooxygenase-2 and transcellular processing: A novel mechanism for NSAID and omega-3 PUFA therapeutic actions. Journal of Physiology and Pharmacology 4: 643-654.

118. Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N. & Gronert, K. (2000) Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. Journal of Experimental Medicine 192: 1197-1204.

119. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G. & Moussignac, R. L. (2002) Resolvins: A family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. Journal of Experimental Medicine 196: 1025-1037.

120. Serhan, C. N., Arita, M., Hong, S. & Gotlinger, K. (2004) Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. Lipids 39: 1125-1132.

121. Calder, P. C., Yaqoob, P., Thies, F., Wallace, F. A. & Miles, E. A. (2002) Fatty acids and lymphocyte functions. British Journal of Nutrition 87: S31-S48.

122. Soyland, E., Nenseter, M. S., Braathen, L. & Drevon, C. A. (1993) Very long chain n-3 and n-6 polyunsaturated fatty acids inhibit proliferation of human T-lymphocytes in vitro. European Journal of Clinical Investigation 23: 112-121.

123. Khalfoun, B., Lacord, M., Bardos, P. & Lebranchu, Y. (1996) Effects of docosahexaenoic and eicosapentaenoic acids on in vitro- induced human lymphoproliferative responses. Transplantation Proceedings 28: 2913-2914.

124. Khalfoun, B., Thibault, G., Lacord, M., Gruel, Y., Bardos, P. & Lebranchu, Y. (1996) Docosahexaenoic and eicosapentaenoic acids inhibit human lymphoproliferative responses in vitro but not the expression of T cell surface activation markers. Scandinavian Journal of Immunology 43: 248-256.

125. Purasiri, P., Mckechnie, A., Heys, S. D. & Eremin, O. (1997) Modulation in vitro of human natural cytotoxicity, lymphocyte proliferative response to mitogensy and cytokine production by essential fatty acids. Immunology 92: 166-172.

126. Calder, P. C., Bevan, S. J. & Newsholme, E. A. (1992) The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. Immunology 75: 108-115.

127. Alexander, N. J. & Smythe, N. L. (1988) Dietary fat modulation of in vitro lymphocyte function. Annals of Nutrition and Metabolism 32: 192-199.

128. Jolly, C. A., Jiang, Y.-H., Chapkin, R. S. & McMurray, D. N. (1997) Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. Journal of Nutrition 127: 37-43.

129. Wallace, F. A., Miles, E. A., Evans, C., Stock, T. E., Yaqoob, P. & Calder, P. C. (2001) Dietary fatty acids influence the production of Th1- but not Th2-type cytokines. Journal of Leukocyte Biology 69: 449-457.

130. Sanderson, P., Yaqoob, P. & Calder, P. C. (1995) Effects of dietary lipid manipulation upon rat spleen lymphocyte functions and the expression of lymphocyte surface molecules. Journal of Nutritional and Environmental Medicine 5: 119-132.

131. Yaqoob, P., Newsholme, E. A. & Calder, P. C. (1994) The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. Immunology 82: 603-610.

132. Meydani, S. N., Lichtenstein, A. H., Cornwall, S., Meydani, M., Goldin, B. R., Rasmussen, H., Dinarello, C. A. & Schaefer, E. J. (1993) Immunologic effects of national cholesterol education panel step-2 diets with and without fish-derived N-3 fatty acid enrichment. Journal of Clinical Investigation 92: 105-113.

133. Molvig, J., Pociot, F., Worsaae, H., Wogensen, L. D., Baek, L., Christensen, P., Mandrup-Poulsen, T., Andersen, K., Madsen, P. et al. (1991) Dietary supplementation with omega-3-polyunsaturated fatty acids decreases mononuclear cell proliferation and interleukin-1beta content but not monokine secretion in healthy and insulin-dependent diabetic individuals. Scandinavian Journal of Immunology 34: 399-410.

134. Gallai, V., Sarchielli, P., Trequattrini, A., Franceschini, M., Floridi, A., Firenze, C., Alberti, A., Di, B. D. & Stragliotto, E. (1995) Cytokine secretion and eicosanoid production in the peripheral blood mononuclear cells of MS patients undergoing dietary supplementation with omega-3 polyunsaturated fatty acids. Journal of Neuroimmunology. 56: 143-153.

135. Endres, S., Meydani, S. N., Ghorbani, R., Schindler, R. & Dinarello, C. A. (1993) Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. Journal of Leukocyte Biology 54: 599-603.

136. Kelley, D. S., Taylor, P. C., Nelson, G. J. & Mackey, B. E. (1998) Dietary docosahexaenoic acid and immunocompetence in young healthy men. Lipids 33: 559-566.

137. Adam, O., Beringer, C., Kless, T., Lemmen, C., Adam, A., Wiseman, M., Adam, P., Klimmek, R. & Forth, W. (2003) Anti-inflammatory effects of a low arachidonic acid diet and fish oil in patients with rheumatoid arthritis. Rheumatology International 23: 27-36.

138. Volker, D., Fitzgerald, P., Major, G. & Garg, M. (2000) Efficacy of fish oil concentrate in the treatment of rheumatoid arthritis. Journal of Rheumatology 27: 2343-2346.

139. Kremer, J. M., Lawrence, D. A., Petrillo, G. F., Litts, L. L., Mullaly, P. M., Rynes, R. I., Stocker, R. P., Parhami, N., Greenstein, N. S. & Fuchs, B. R. (1995) Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. Arthritis & Rheumatism 38: 1107-1114.

140. Aslan, A. & Triadafilopoulos, G. (1992) Fish oil fatty acid supplementation in active ulcerative colitis: a double-blind, placebo-controlled, crossover study. American Journal of Gastroenterology 87: 432-437.

141. Lorenz, R., Weber, P. C., Szimnau, P., Heldwein, W., Strasser, T. & Loeschke, K. (1989) Supplementation with n-3 fatty acids from fish oil in chronic inflammatory bowel disease-a randomized, placebo-controlled, double-blind cross-over trial. Journal of Internal Medicine 731: S225-S232.

142. Nordvik, I., Myhr, K. M., Nyland, H. & Bjerve, K. S. (2000) Effect of dietary advice and n-3 supplementation in newly diagnosed MS patients. Acta Neurologica Scandinavica 102: 143-149.

143. Duffy, E. M., Meenagh, G. K., McMillan, S. A., Strain, J. J., Hannigan, B. M. & Bell, A. L. (2004) The clinical effect of dietary supplementation with omega-3 fish oils and/or copper in systemic lupus erythematosus. Journal of Rheumatology 31: 1551-1561.

144. Ilowite, N. T., Copperman, N., Leicht, T., Kwong, T. & Jacobson, M. S. (1995) Effects of dietary modification and fish oil supplementation on dyslipoproteinemia in pediatric systemic lupus erythematosus. Journal of Rheumatology 22: 1347-1351.

145. Miles, E. A., Aston, L. & Calder, P. C. (2003) In vitro effects of eicosanoids derived from different 20-carbon fatty acids on T helper type 1 and T helper type 2 cytokine production in human whole-blood cultures. Clinical & Experimental Allergy 33: 624-632.

146. Roper, R. L. & Phipps, R. P. (1994) Prostaglandin E2 regulation of the immune response. Advances in Prostaglandin, Thromboxane, & Leukotriene Research 22: 101-111.

147. Payan, D. G., Wong, M. Y., Chernov-Rogan, T., Valone, F. H., Pickett, W. C., Blake, V. A., Gold, W. M. & Goetzl, E. J. (1986) Alterations in human leukocyte function induced by ingestion of eicosapentaenoic acid. Journal of Clinical Immunology 6: 402-410.

148. Arm, J. P., Horton, C. E., Mencia-Huerta, J. M., House, F., Eiser, N. M., Clark, T. J., Spur, B. W. & Lee, T. H. (1988) Effect of dietary supplementation with fish oil lipids on mild asthma. Thorax 43: 84-92.

149. Kirsch, C. M., Payan, D. G., Wong, M. Y., Dohlman, J. G., Blake, V. A., Petri, M. A., Offenberger, J., Goetzl, E. J. & Gold, W. M. (1988) Effect of eicosapentaenoic acid in asthma. Clinical Allergy 18: 177-187.

150. Schachter, H. M., Reisman, J., Tran, K., Dales, B., Kourad, K., Barnes, D., Sampson, M., Morrison, A., Gaboury, I. & Blackman, J. (2004) Health effects of omega-3 fatty acids on asthma. Evidence Report: Technology Assessment 91: 1-7.

151. Woods, R. K., Thien, F. C. & Abramson, M. J. (2002) Dietary marine fatty acids (fish oil) for asthma in adults and children. Cochrane Database of Systematic Reviews. (3):CD001283.

152. Broughton, K. S., Johnson, C. S., Pace, B. K., Liebman, M. & Kleppinger, K. M. (1997) Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. American Journal of Clinical Nutrition 65: 1011-1017.

153. Yu, G. & Bjorksten, B. (1998) Polyunsaturated fatty acids in school children in relation to allergy and serum IgE levels. Pediatric Allergy and Immunology 9: 133-138.

154. Sausenthaler, S., Koletzko, S., Schaaf, B., Lehmann, I., Borte, M., Herbarth, O., von, B. A., Wichmann, H. E. & Heinrich, J. (2007) Maternal diet during pregnancy in relation to eczema and allergic sensitization in the offspring at 2 y of age. American Journal of Clinical Nutrition 85: 530-537.

155. Romieu, I., Torrent, M., Garcia-Esteban, R., Ferrer, C., Ribas-Fito, N., Anto, J. M. & Sunyer, J. (2007) Maternal fish intake during pregnancy and atopy and asthma in infancy. Clinical and Experimental Allergy 37: 518-525.

156. Willers, S., Devereux, G., Craig, L., McNeill, G., Wijga, A., bou El-Magd, W., Turner, S., Helms, P. & Seaton, A. (2007) Maternal food consumption during pregnancy and asthma, respiratory and atopic symptoms in 5-year-old children. Thorax 62: 773-779.

157. Salam, M. T., Li, Y. F., Langholz, B. & Gilliland, F. D. (2005) Maternal fish consumption during pregnancy and risk of early childhood asthma. Journal of Asthma 42: 513-518.

158. Calvani, M., Alessandri, C., Sopo, S. M., Panetta, V., Pingitore, G., Tripodi, S., Zappala, D. & Zicari, A. M. (2006) Consumption of fish, butter and margarine during pregnancy and development of allergic sensitizations in the offspring: role of maternal atopy. Pediatric Allergy and Immunology 17: 94-102.

159. Hoppu, U., Rinne, M., Lampi, A. M. & Isolauri, E. (2005) Breast milk fatty acid composition is associated with development of atopic dermatitis in the infant. Journal of Pediatric Gastroenterology and Nutrition 41: 335-338.

160. Martin, J. C., Bougnoux, P., Fignon, A., Theret, V., Antoine, J. M., Lamisse, F. & Couet, C. (1993) Dependence of human milk essential fatty acids on adipose stores during lactation. American Journal of Clinical Nutrition 58: 653-659.

161. Demmelmair, H., Sauerwald, T., Fidler, N., Baumheuer, M. & Koletzko, B. (2001) Polyunsaturated fatty acid metabolism during lactation. World Review of Nutrition & Dietetics 88: 184-189.

162. Hodge, L., Salome, C. M., Peat, J. K., Haby, M. M., Xuan, W. & Woolcock, A. J. (1996) Consumption of oily fish and childhood asthma risk. Medical Joutrnal of Australia 164: 137-140.

163. Dunder, T., Kuikka, L., Turtinen, J., Rasanen, L. & Uhari, M. (2001) Diet, serum fatty acids, and atopic diseases in childhood. Allergy 56: 425-428.

164. Nafstad, P., Nystad, W., Magnus, P. & Jaakkola, J. J. (2003) Asthma and allergic rhinitis at 4 years of age in relation to fish consumption in infancy. Journal of Asthma 40: 343-348.

165. Andreasyan, K., Ponsonby, A. L., Dwyer, T., Kemp, A., Dear, K., Cochrane, J. & Carmichael, A. (2005) A differing pattern of association between dietary fish and allergen-specific subgroups of atopy. Allergy 60: 671-677.

166. Kim, J. L., Elfman, L., Mi, Y., Johansson, M., Smedje, G. & Norback, D. (2005) Current asthma and respiratory symptoms among pupils in relation to dietary factors and allergens in the school environment. International Journal of Indoor Environment and Health 15: 170-182.

167. Kull, I., Bergstrom, A., Lilja, G., Pershagen, G. & Wickman, M. (2006) Fish consumption during the first year of life and development of allergic diseases during childhood. Allergy 61: 1009-1015.

168. Chatzi, L., Torrent, M., Romieu, I., Garcia-Esteban, R., Ferrer, C., Vioque, J., Kogevinas, M. & Sunyer, J. (2007) Diet, wheeze, and atopy in school children in Menorca, Spain. Pediatric Allergy and Immunology 18: 480-485.

169. Antova, T., Pattenden, S., Nikiforov, B., Leonardi, G. S., Boeva, B., Fletcher, T., Rudnai, P., Slachtova, H., Tabak, C. et al. (2003) Nutrition and respiratory health in children in six Central and Eastern European countries. Thorax 58: 231-236.

170. Huang, S. L., Lin, K. C. & Pan, W. H. (2001) Dietary factors associated with physician-diagnosed asthma and allergic rhinitis in teenagers: analyses of the first Nutrition and Health Survey in Taiwan. Clinical and Experimental Allergy 31: 259-264.

171. Takemura, Y., Sakurai, Y., Honjo, S., Tokimatsu, A., Gibo, M., Hara, T., Kusakari, A. & Kugai, N. (2002) The relationship between fish intake and the prevalence of asthma: The Tokorozawa childhood asthma and pollinosis study. Preventive Medicine 34: 221-225.

172. Hijazi, N., Abalkhail, B. & Seaton, A. (2000) Diet and childhood asthma in a society in transition: A study in urban and rural Saudi Arabia. Thorax 55: 775-779.

173. Farchi, S., Forastiere, F., Agabiti, N., Corbo, G., Pistelli, R., Fortes, C., Dell'Orco, V. & Perucci, C. A. (2003) Dietary factors associated with wheezing and allergic rhinitis in children. European Respiratory Journal 22: 772-780.

174. Wijga, A. H., Smit, H. A., Kerkhof, M., de Jongste, J. C., Gerritsen, J., Neijens, H. J., Boshuizen, H. C. & Brunekreef, B. (2003) Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. Thorax 58: 567-572.

175. Alm, B., Aberg, N., Erdes, L., Mollborg, P., Pettersson, R., Norvenius, S. G., Goksor, E. & Wennergren, G. (2009) Early introduction of fish decreases the risk of eczema in infants. Archives of Disease in Childhood 94: 11-15.

176. Barden, A. E., Mori, T. A., Dunstan, J. A., Taylor, A. L., Thornton, A. C. A., Croft, K. D., Beilin, L. J. & Prescott, S. L. (2004) Fish oil supplementation in pregnancy lowers F2-isoprostanes in neonates at high risk of atopy. Free Radical Research 38: 233-239.

177. Denburg, J. A., Hatfield, H. M., Cyr, M. M., Hayes, L., Holt, P. G., Sehmi, R., Dunstan, J. A. & Prescott, S. L. (2005) Fish oil supplementation in pregnancy modifies neonatal progenitors at birth in infants at risk of atopy. Pediatric Research 57: 276-281.

178. Dunstan, J. A., Mori, T. A., Bardent, A., Beilin, L. J., Taylor, A. L., Holt, P. G. & Prescott, S. L. (2003) Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clinical and Experimental Allergy 33: 442-448.

179. Dunstan, J. A., Roper, J., Mitoulas, L., Hartmann, P. E., Simmer, K. & Prescott, S. L. (2004) The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition. Clinical and Experimental Allergy 34: 1237-1242.

180. Dunstan, J. A., Mori, T. A., Barden, A., Beilin, L. J., Holt, P. G., Calder, P. C., Taylor, A. L. & Prescott, S. L. (2004) Effects of omega-3 polyunsaturated fatty acid supplementation in pregnancy on maternal and fetal erythrocyte fatty composition. European Journal of Clinical Nutrition 58: 429-437.

181. Dunstan, J. A., Mitoulas, L. R., Dixon, G., Doherty, D. A., Hartmann, P. E., Simmer, K. & Prescott, S. L. (2007) The effects of fish oil supplementation in pregnancy on breast milk fatty acid composition over the course of lactation: a randomized controlled trial. Pediatric Research 62: 689-694.

182. Prescott, S. L., Barden, A. E., Mori, T. A. & Dunstan, J. A. (2007) Maternal fish oil supplementation in pregnancy modifies neonatal leukotriene production by cord-blood-derived neutrophils. Clinical Science 113: 409-416.

183. Krauss-Etschmann, S., Hartl, D., Rzehak, P., Heinrich, J., Shadid, R., Del, C. R.-T., Campoy, C., Pardillo, S., Schendel, D. J. et al. (2008) Decreased cord blood IL-4, IL-13, and CCR4 and increased TGF-beta levels after fish oil supplementation of pregnant women. Journal of Allergy & Clinical Immunology 121: 464-470.

184. Krauss-Etschmann, S., Shadid, R., Campoy, C., Hoster, E., Demmelmair, H., Jimenez, M., Gil, A., Rivero, M., Veszpremi, B. et al. (2007) Effects of fish-oil and folate supplementation of pregnant women on maternal and fetal plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid: A European randomized multicenter trial. American Journal of Clinical Nutrition 85: 1392-1400.

185. Lauritzen, L., Kjaer, T. M. R., Fruekilde, M.-B., Michaelsen, K. F. & Frokiaer, H. (2005) Fish oil supplementation of lactating mothers affects cytokine production in 2 1/2-year-old children. Lipids 40: 669-676.

186. Olsen, S. F., Osterdal, M. L., Salvig, J. D., Mortensen, L. M., Rytter, D., Secher, N. J. & Henriksen, T. B. (2008) Fish oil intake compared with olive oil intake in late pregnancy and asthma in the

offspring: 16 y of registry-based follow-up from a randomized controlled trial. American Journal of Clinical Nutrition 88: 167-175.

187. Furuhjelm, C., Warstedt, K., Larsson, J., Fredriksson, M., Fageras Bottcher, M., Falth-Magnusson, K. & Duchen, K. (2009) Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy. Acta Paediatrica 98: 1461-1467.

188. Warstedt, K., Furuhjelm, C., Duchen, K., Falth-Magnusson, K. & Fageras, M. (2009) The effects of omega-3 fatty acid supplementation in pregnancy on maternal eicosanoid, cytokine, and chemokine secretion. Pediatric Research 2: 212-217.

189. Larque, E., Krauss-Etschmann, S., Campoy, C., Hartl, D., Linde, J., Klingler, M., Demmelmair, H., Cano, A., Gil, A. et al. (2006) Docosahexaenoic acid supply in pregnancy affects placental expression of fatty acid transport proteins. American Journal of Clinical Nutrition 84: 853-861.

190. Mihrshahi, S., Peat, J. K., Webb, K., Tovey, E. R., Marks, G. B., Mellis, C. M. & Leeder, S. R. (2001) The Childhood Asthma Prevention Study (CAPS): Design and research protocol of a randomized trial for the primary prevention of asthma. Controlled Clinical Trials 22: 333-354.

191. Mihrshahi, S., Peat, J. K., Marks, G. B., Mellis, C. M., Tovey, E. R., Webb, K., Britton, W. J. & Leeder, S. R. (2003) Eighteen-month outcomes of house dust mite avoidance and dietary fatty acid modification in the Childhood Asthma Prevention Study (CAPS). Journal of Allergy and Clinical Immunology 111: 162-168.

192. Mihrshahi, S., Peat, J. K., Webb, K., Oddy, W., Marks, G. B. & Mellis, C. M. (2004) Effect of omega-3 fatty acid concentrations in plasma on symptoms of asthma at 18 months of age. Pediatric Allergy and Immunology 15: 517-522.

193. Peat, J. K., Mihrshahi, S., Kemp, A. S., Marks, G. B., Tovey, E. R., Webb, K., Mellis, C. M. & Leeder, S. R. (2004) Three-year outcomes of dietary fatty acid modification and house dust mite reduction in the Childhood Asthma Prevention Study. Journal of Allergy and Clinical Immunology 114: 807-813.

194. Marks, G. B., Mihrshahi, S., Kemp, A. S., Tovey, E. R., Webb, K., Almqvist, C., Ampon, R. D., Crisafulli, D., Belousova, E. G. et al. (2006) Prevention of asthma during the first 5 years of life: A randomized controlled trial. Journal of Allergy and Clinical Immunology 118: 53-61.

195. Almqvist, C., Garden, F., Xuan, W., Mihrshahi, S., Leeder, S. R., Oddy, W., Webb, K. & Marks, G. B. (2007) Omega-3 and omega-6 fatty acid exposure from early life does not affect atopy and asthma at age 5 years. Journal of Allergy and Clinical Immunology 119: 1438-1444.

196. Damsgaard, C. T., Lauritzen, L., Kjaer, T. M. R., Holm, P. M. I., Fruekilde, M.-B., Michaelsen, K. F. & Frokiaer, H. (2007) Fish oil supplementation modulates immune function in healthy infants. Journal of Nutrition 137: 1031-1036.

197. Hodge, L., Salome, C. M., Hughes, J. M., Liu-Brennan, D., Rimmer, J., Allman, M., Pang, D., Armour, C. & Woolcock, A. J. (1998) Effect of dietary intake of omega-3 and omega-6 fatty acids on severity of asthma in children. European Respiratory Journal 11: 361-365.

198. Nagakura, T., Matsuda, S., Shichijyo, K., Sugimoto, H. & Hata, K. (2000) Dietary supplementation with fish oil rich in omega-3 polyunsaturated fatty acids in children with bronchial asthma. European Respiratory Journal 16: 861-865.

199. Vaisman, N., Zaruk, Y., Shirazi, I., Kaysar, N. & Barak, V. (2005) The effect of fish oil supplementation on cytokine production in children. European Cytokine Network 16: 194-198.

200. Damsgaard, C. T., Schack-Nielsen, L., Michaelsen, K. F., Fruekilde, M.-B., Hels, O. & Lauritzen, L. (2006) Fish oil affects blood pressure and the plasma lipid profile in healthy Danish infants. Journal of Nutrition 136: 94-99.

201. Inskip, H. M., Godfrey, K. M., Robinson, S. M., Law, C. M., Barker, D. J., Cooper, C. & SWS Study Group (2006) Cohort profile: The Southampton Women's Survey. International Journal of Epidemiology 35: 42-48.

202. Inskip, H. M., Godfrey, K. M., Martin, H. J., Simmonds, S. J., Cooper, C., Sayer, A. A. & Southampton Women's Survey Study Group (2007) Size at birth and its relation to muscle strength in young adult women. Journal of Internal Medicine 262: 368-374.

203. Harvey, N. C., Poole, J. R., Javaid, M. K., Dennison, E. M., Robinson, S., Inskip, H. M., Godfrey, K. M., Cooper, C., Sayer, A. A. & SWS Study Group (2007) Parental determinants of neonatal body composition. Journal of Clinical Endocrinology & Metabolism 92: 523-526.

204. WHO (1989) Toxicological evaluation of certain food additives and contaminants. Cambridge, Cambrigde University Press (WHO Food Additives, No 24).

205. FAO/WHO (2003) Safety evaluation of certain food additives and contaminants; methylmercury. Summary and conclusions of the 61st JECFA meeting.

206. Innis, S. M. (2008) Dietary omega 3 fatty acids and the developing brain. Brain Research 1237: 35-43.

207. Javaid, M. K., Crozier, S. R., Harvey, N. C., Gale, C. R., Dennison, E. M., Boucher, B. J., Arden, N. K., Godfrey, K. & Cooper, C. (2006) Maternal vitamin D status during pregancy and childhood bone mass at age 9 years: a longitudinal study. The Lancet 367: 36-43.

208. Cole, Z. A., Gale, C. R., Javaid, M. K., Robinson, S. M., Law, C., Boucher, B. J., Crozier, S. R., Godfrey, K. M., Dennison, E. M. & Cooper, C. (2009) Maternal dietary patterns during pregancy and childhood bone mass: a longitudinal study. Journal of Bone and Mineral Research 24: 663-668.

209. Nwaru, B. I., Ahonen, S., Kaila, M., Erkkola, M., Haapala, A.-M., Kronberg-Kippila, C., Veijola, R., Ilonen, J., Simell, O. et al. (2010) Maternal diet during pregnancy and allergic sensitisation in the offspring by 5 years of age: a prospective cohort study. Pediatric Allergy & Immunology 21: 29-37.

210. Devereux, G., MacDonald, H. & Hawrylowicz, C. (2009) Vitamin D and asthma: time for intervention? American Journal of Respiratory & Critical Care Medicine 179: 739-740.

211. Devereux, G., Litonjua, A. A., Turner, S. W., Craig, L. C., McNeill, G., Martindale, S., Helms, P. J., Seaton, A. & Weiss, S. T. (2007) Maternal vitamin D intake during pregnancy and early childhood wheezing. American Journal of Clinical Nutrition 85: 853-859.

212. Erkkola, M., Kaila, M., Nwaru, B. I., Kronberg-Kippila, C., Ahonen, S., Nevalainen, J., Veijola, R., Pekkanen, J., Ilonen, J. et al. (2009) Maternal vitamin D intake during pregnancy is inversely associated with asthma and allergic rhinitis in 5-year-old children. Clinical & Experimental Allergy 39: 875-882.

213. Devereux, G., McNeill, G., Newman, G., Turner, S., Craig, L., Martindale, S., Helms, P. & Seaton, A. (2007) Early childhood wheezing symptoms in relation to plasma selenium in pregnant mothers and neonates. Clinical & Experimental Allergy 37: 1000-1008.

214. Crozier, S. R., Inskip, H. M., Godfrey, K. M. & Robinson, S. M. (2008) Dietary patterns in pregnant women: a comparison of food-frequency questionnaires and 4 d prospective diaries. British Journal of Nutrition 99: 869-875.

215. Borland, S. E., Robinson, S. M., Crozier, S. R., Inskip, H. M. & SWS Study Group (2008) Stability of dietary patterns in young women over a 2-year period. European Journal of Clinical Nutrition 62: 119-126.

216. Crozier, S. R., Robinson, S. M., Borland, S. E., Inskip, H. M. & SWS Study Group (2006) Dietary patterns in the Southampton Women's Survey. European Journal of Clinical Nutrition 60: 1391-1399.

217. Robinson, S., Godfrey, K., Osmond, C., Cox, V. & Barker, D. (1996) Evaluation of a food frequency questionnaire used to assess nutrient intakes in pregnant women. European Journal of Clinical Nutrition 50: 302-308.

218. Crozier, S. R., Robinson, S. M., Godfrey, K. M., Cooper, C. & Inskip, H. M. (2009) Women's dietary patterns change little from before to during pregnancy. Journal of Nutrition 139: 1956-1963.

219. Godfrey, K., Robinson, S., Barker, D. J., Osmond, C. & Cox, V. (1996) Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. British Medical Journal 312: 410-414.

220. Kremmyda, L. S., Papadaki, A., Hondros, G., Kapsokefalou, M. & Scott, J. A. (2008) Differentiating between the effect of rapid dietary acculturation and the effect of living away from home for the first time, on the diets of Greek students studying in Glasgow. Appetite 50: 455-463.

221. Crawley, H. (1988) Food portion sizes. London: HMSO.

222. British Nutrition Foundation (BNF) (2001) Selenium and Health. Briefing paper.

223. Kant, A. K. (2004) Dietary patterns and health outcomes. Journal of American Dietetic Association 104: 615-635.

224. Joliffe, I. T. & Morgan, B. J. T. (1992) Principal component analysis and exploratory factor analysis. Statistical Methods in Medical Research 1: 69-95.

225. Northstone, K. & Emmett, P. M. (2008) A comparison of methods to assess changes in dietary patterns from pregnancy to 4 years post-partum obtained using principal components analysis. British Journal of Nutrition 99: 1099-1106.

226. Mishra, G. D., McNaughton, S. A., Bramwell, G. D. & Wadsworth, M. E. J. (2006) Longitudinal changes in dietary patterns during adult life. British Journal of Nutrition 96: 735-744.

227. Slattery, M. L., Boucher, K. M., Caan, B. J., Potter, J. D. & Ma, K.-N. (1998) Eating patterns and risk of colon cancer. American Journal of Epidemiology 148: 4-16.

228. Department of Health (1998) Committee on Medical Aspects of Food Policy. Nutritional Aspects of the Development of Cancer. The Stationary Office, London.

229. Crozier, S. R., Robinson, S. M., Borland, S. E., Godfrey, K. M., Cooper, C. & Inskip, H. M. (2009) Do women change their health behaviours in pregnancy? Findings from the Southampton Women's Survey. Paediatric and Perinatal Epidemiology 23: 446-453.

230. Kipnis, V., Subar, A. F., Midthune, D., Freedman, L. S., Ballard-Barbash, R., Troiano, R. P., Bingham, S., Schoeller, D. A., Schatzkin, A. & Carroll, R. J. (2003) Structure of dietary measurement error: Results of the OPEN biomarker study. American Journal of Epidemiology 158: 14-21.

231. Schatzkin, A., Kipnis, V., Carroll, R. J., Midthune, D., Subar, A. F., Bingham, S., Schoeller, D. A., Troiano, R. P. & Freedman, L. S. (2003) A comparison of a food frequency questionnaire with a 24-hour recall for use in an epidemiological cohort study: Results from the biomarker-based Observing Protein and Energy Nutrition (OPEN) study. International Journal of Epidemiology 32: 1054-1062.

232. Ortiz-Andrellucchi, A., Doreste-Alonso, J., Henriquez-Sanchez, P., Cetin, I. & Serra-Majem, L. (2009) Dietary assessment methods for micronutrient intake in pregnant women: a systematic review. British Journal of Nutrition 102: S64-S86.

233. Mikkelsen, T. B., Olser, M. & Olsen, S. F. (2006) Validity of protein, retinol, folic acid and n-3 fatty acid intakes estimated from the food-frequency questionnaire used in the Danish National Birth Cohort. Public Health Nutrition 9: 771-778.

234. Sullivan, B. L., Bown, J., Williams, P. G. & Meyer, B. J. (2008) Dietary validtion of a new Australian food-frequency questionnaire that estimates long-chain n-3 polyunsaturated fatty acids. British Journal of Nutrition 99: 666.

235. McNaughton, S. A., Hughes, M. C. & Marks, G. C. (2007) Validation of a FFQ to estimate the intake of PUFA using plasma phospholipid fatty acids and weighed foods records. British Journal of Nutrition 97: 561-568.

236. Overby, N. C., Serra-Majem, L. & Andersen, L. F. (2009) Dietary assessment methods on n-3 fatty acid intake: a systematic review. British Journal of Nutrition 102: S56-S63.

237. Crozier, S. R., Inskip, H. M., Barker, M. E., Lawerence, W. T., Cooper, C., Robinson, S. M. & the SWS Study Group (2010) Development of a 20-item food frequency questionnaire to assess a 'prudent' dietary pattern among young women in Southampton. European Journal of Clinical Nutrition 64: 99-104.

238. Din, J. N., Harding, S. A., Valerio, C. J., Sarma, J., Lyall, K., Riemersma, R. A., Newby, D. E. & Flapan, A. D. (2008) Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man. Atherosclerosis 197: 290-296.

239. Gunnarsdottir, I., Tomasson, H., Kiely, M., Martinez, J. A., Bandarra, N. M., Morais, M. G. & Thorsdottir, I. (2008) Inclusion of fish or fish oil in weight-loss diets for young adults: Effects on blood lipids. International Journal of Obesity 32: 1105-1112.

240. Navas-Carretero, S., Perez-Granados, A. M., Schoppen, S., Sarria, B., Carbajal, A. & Vaquero, M. P. (2009) Iron status biomarkers in iron deficient women consuming oily fish versus red meat diet. Journal of Physiology and Biochemistry 65: 165-174.

241. Pot, G. K., Majsak-Newman, G., Geelen, A., Harvey, L. J., Nagengast, F. M., Witteman, B. J. M., Van De Meeberg, P. C., Timmer, R., Tan, A. et al. (2009) Fish consumption and markers of colorectal cancer risk: A multicenter randomized controlled trial. American Journal of Clinical Nutrition 90: 354-361.

242. Pot, G. K., Geelen, A., Majsak-Newman, G., Harvey, L. J., Nagengast, F. M., Witteman, B. J., Van De Meeberg, P. C., Hart, A. R., Schaafsma, G. et al. (2010) Increased consumption of fatty and lean fish reduces serum C-reactive protein concentrations but not inflammation markers in feces and in colonic biopsies. Journal of Nutrition 140: 371-376.

243. Vidgren, H. M., Agren, J. J., Schwab, U., Rissanen, T., Hanninen, O. & Uusitupa, M. I. J. (1997) Incorporation of n-3 fatty acids into plasma lipid fractions, and erythrocyte membranes and platelets during dietary supplementation with fish, fish oil, and docosahexaenoic acid-rich oil among healthy young men. Lipids 32: 697-705.

244. Lomax, A. R. & Calder, P. C. (2009) Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans. Current Pharmaceutical Design 15: 1428-1518.

245. Haggarty, P. (2004) Effect of placental function in fatty acid requirements during pregnancy. European Journal of Clinical Nutrition 58: 1559-1570.

246. Smuts, C. M., Huang, M., Mundy, D., Plasse, T., Major, S. & Carlson, S. E. (2003) A randomized trial of docosahexaenoic acid supplementation during the third trimester of pregnancy. Obstetrics and Gynecology 101: 469-479.

247. Klingler, M., Demmelmair, H., Larque, E. & Koletzko, B. (2003) Analysis of FA contents in individual lipid fractions from human placental tissue. Lipids 38: 561-566.

248. Hanebutt, F. L., Demmelmair, H., Schiessl, B., Larque, E. & Koletzko, B. (2008) Long-chain polyunsaturated fatty acid (LC-PUFA) transfer across the placenta. Clinical Nutrition 27: 685-693.

249. Olsen, S. F. & Secher, N. J. (2002) Low consumption of seafood in early pregnancy as a risk factor for preterm delivery: prospective cohort study. British Medical Journal 324: 447.

250. Innis, S. M. (1991) Essential fatty acids in growth and development. Progress in Lipid Research 30: 39-103.

251. Koletzko, B., Agostoni, C., Carlson, S. E., Clandinin, T., Hornstra, G., Neuringer, M., Uauy, R., Yamashiro, Y. & Willatts, P. (2001) Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. Acta Paediatrica, International Journal of Paediatrics 90: 460-464.

252. Koletzko, B. & Rodriguez-Palmero, M. (1999) Polyunsaturated fatty acids in human milk and their role in early infant developement. Journal of Mammary Gland Biology and Neoplasia 4: 269-284.

253. Neuringer, M. (2000) Infant vision and retinal function in studies of dietary long-chain polyunsaturated fatty acids: methods, results, and implications. American Journal of Clinical Nutrition 71: S256-S267.

254. Clandinin, M. T., Van Aerde, J. E., Merkel, K. L., Harris, C. L., Springer, M. A., Hansen, J. W. & Diersen-Schade, D. A. (2005) Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid. Journal of Pediatrics 146: 461-468.

255. Herrera, E. (2002) Lipid metabolism in pregnancy and its consequences in the fetus and newborn. Endocrine 19: 43-55.

256. Herrera, E. (2000) Metabolic adaptations in pregnancy and their implications for the availability of substrates to the fetus. European Journal of Clinical Nutrition 54: S47-S51.

257. Herrera, E., Lopez-Soldado, I., Limones, M., Amusquivar, E. & Ramos, M. P. (2006) Lipid metabolism during the perinatal phase, and its implications on postnatal development. International Journal for Vitamin and Nutrition Research 76: 216-224.

258. Herrera, E., Amusquivar, E., Lopez-Soldado, I. & Ortega, H. (2006) Maternal lipid metabolism and placental lipid transfer. Hormone Research 65: S59-S64.

259. Parker, C. R., Deahl, T., Drewry, P. & Hankins, G. (2010) Analysis of the potential for transfer of lipoprotein-cholesterol across the human placenta. Early Human Development 8: 289-295.

260. De Vriese, S. R., Dhont, M. & Christophe, A. B. (2003) FA composition of cholesteryl esters and phospholipids in maternal plasma during pregnancy and at delivery and in cord plasma at birth. Lipids 38: 1-7.

261. Berghaus, T. M., Demmelmair, H. & Koletzko, B. (1998) Fatty acid composition of lipid classes in maternal and cord plasma at birth. European Journal of Pediatrics 157: 763-768.

262. Burdge, G. C., Sherman, R. C., Ali, Z., Wootton, S. A. & Jackson, A. A. (2006) Docosahexaenoic acid is selectively enriched in plasma phospholipids during pregnancy in Trinidadian women - Results of a pilot study. Reproduction Nutrition Development 46: 63-67.

263. Min, Y., Ghebremeskel, K., Crawford, M. A., Nam, J. H., Kim, A., Koo, J. N. & Suzuki, H. (2000) Pregnancy reduces arachidonic and docosahexaenoic in plasma triacylglycerols of Korean women. International Journal for Vitamin & Nutrition Research 70: 70-75.

264. Houwelingen, A. C. V., Foreman, V., Nicolini, U., Nicolaides, K. H., Al, M. D. M., Kester, A. D. M. & Hornstra, G. (1996) Essential fatty acid status of fetal plasma phospholipids: Similar to postnatal values obtained at comparable gestational ages. Early Human Development 46: 141-152.

265. Vlaardingerbroek, H. & Hornstra, G. (2004) Essential fatty acids in erythrocyte phospholipids during pregnancy and at delivery in mothers and their neonates: Comparison with plasma phospholipids. Prostaglandins Leukotrienes and Essential Fatty Acids 71: 363-374.

266. De Vriese, S. R., Matthys, C., De, H. S., De, B. G., Dhont, M. & Christophe, A. B. (2002) Maternal and umbilical fatty acid status in relation to maternal diet. Prostaglandins Leukotrienes and Essential Fatty Acids 67: 389-396.

267. Donahue, S. M. A., Rifas-Shiman, S. L., Olsen, S. F., Gold, D. R., Gillman, M. W. & Oken, E. (2009) Associations of maternal prenatal dietary intake of n-3 and n-6 fatty acids with maternal and umbilical cord blood levels. Prostaglandins Leukotrienes and Essential Fatty Acids 80: 289-296.

268. Kuriki, K., Nagaya, T., Tokudome, Y., Imaeda, N., Fujiwara, N., Sato, J., Goto, C., Ikeda, M., Maki, S. et al. (2003) Plasma Concentrations of (n-3) Highly Unsaturated Fatty Acids Are Good Biomarkers of Relative Dietary Fatty Acid Intakes: A Cross-Sectional Study. Journal of Nutrition 133: 3643-3650.

269. Astorg, P., Bertrais, S., Laporte, F., Arnault, N., Estaquio, C., Galan, P., Favier, A. & Hercberg, S. (2008) Plasma n-6 and n-3 polyunsaturated fatty acids as biomarkers of their dietary intakes: A cross-sectional study within a cohort of middle-aged French men and women. European Journal of Clinical Nutrition 62: 1155-1161.

270. Al, M. D. M., van Houwelingen, A. C., Kester, A. D. M., Hasaart, T. H. M., De Jong, A. E. P. & Hornstra, G. (1995) Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. British Journal of Nutrition 74: 55-68.

271. Otto, S. J., Houwelingen, A. C., Antal, M., Manninen, A., Godfrey, K., Lopez-Jaramillo, P. & Hornstra, G. (1997) Maternal and neonatal essential fatty acid status in phospholipids: An international comparative study. European Journal of Clinical Nutrition 51: 232-242.

272. Lauritzen, L., Jorgensen, M. H., Hansen, H. S. & Michaelsen, K. F. (2002) Fluctuations in human milk long-chain PUFA levels in relation to dietary fish intake. Lipids 37: 237-244.

273. Matorras, R., De Larrucea, A. L., Sanjurjo, P., Ruiz, J. I., Echevarria, Y., Nieto, A., Perteagudo, L. & miz-Echvarria, J. L. (2001) Increased tissue concentrations of arachidonic acid in umbilical artery and placenta in fetal growth retardation. Acta Obstetricia et Gynecologica Scandinavica 80: 807-812.

274. Zhang, J., Wang, Y., Meng, L., Wang, C., Zhao, W., Chen, J., Ghebremeskel, K. & Crawford, M. A. (2009) Maternal and neonatal plasma n-3 and n-6 fatty acids of pregnant women and neonates in three regions in China with contrasting dietary patterns. Asia Pacific Journal of Clinical Nutrition 18: 377-388.

275. Damsgaard, C. T., Frokiaer, H. & Lauritzen, L. (2008) The effects of fish oil and high or low linoleic acid intake on fatty acid composition of human peripheral blood mononuclear cells. British Journal of Nutrition 99: 147-154.

276. Hawkes, J. S., Bryan, D.-L., Makrides, M., Neumann, M. A. & Gibson, R. A. (2002) A randomized trial of supplementation with docosahexaenoic acid-rich tuna oil and its effects on the human milk cytokines interleukin 1beta, interleukin 6, and tumor necrosis factor alpha. American Journal of Clinical Nutrition 75: 754-760.

277. Wegmann, T. G., Lin, H., Guilbert, L. & Mosmann, T. R. (1993) Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology Today 14: 353-356.

278. Vince, G. S. & Johnson, P. M. (1996) Is there a Th2 bias in human pregnancy? Journal of Reproductive Immunology 32: 101-104.

279. Chaouat, G., Ledee-Bataille, N., Dubanchet, S., Zourbas, S., Sandra, O. & Martal, J. (2004) Th1/Th2 paradigm in pregnancy: Paradigm lost? Cytokines in pregnancy/early abortion: Reexamining the Th1/Th2 paradigm. International Archives of Allergy and Immunology 2004: 93-119.

280. Piccinni, M. P., Maggi, E. & Romagnani, S. (2000) Role of hormone-controlled T-cell cytokines in the maintenance of pregnancy. Biochemical Society Transactions 28: 212-215.

281. Borzychowski, A. M., Croy, B. A., Chan, W. L., Redman, C. W. & Sargent, I. L. (2005) Changes in systemic type 1 and type 2 immunity in normal pregnancy and pre-eclampsia may be mediated by natural killer cells. European Journal of Immunology 35: 3054-3063.

282. Kwak-Kim, J. & Gilman-Sachs, A. (2008) Clinical implication of natural killer cells and reproduction. American Journal of Reproductive Immunology 59: 388-400.

283. Thornton, C. A., Holloway, J. A., Shute, J. K., Holloway, J. W., Diaper, N. D. & Warner, J. O. (2009) Human mid-gestation amniotic fluid contains interleukin-16 bioactivity. Immunology 126: 543-551.

284. Bertino, E., Bisson, C., Martano, C., Coscia, A., Fabris, C., Monti, G., Testa, T. & Conti, A. (2006) Relationship between maternal- and fetal-specific IgE. Pediatric Allergy & Immunology 17: 484-488.

285. Larsson, A. K., Nilsson, C., Hoglind, A., Sverremark-Ekstrom, E., Lilja, G. & Troye-Blomberg, M. (2006) Relationship between maternal and child cytokine responses to allergen and phytohaemagglutinin 2 years after delivery. Clinical & Experimental Immunology 144: 401-408.

286. Prescott, S. L., Taylor, A., Roper, J., Wahdan, A., Noakes, P., Thornton, C., Dunstan, J. & Upham, J. W. (2005) Maternal reactivity to fetal alloantigens is related to newborn immune responses and subsequent allergic disease. Clinical & Experimental Allergy 35: 417-425.

287. Breckler, L. A., Hale, J., Jung, W., Westcott, L., Dunstan, J. A., Thornton, C. A. & Prescott, S. L. (2010) Modulation of in vivo and in vitro cytokine production over the course of pregnancy in allergic and non-allergic mothers. Pediatric Allergy & Immunology 21: 14-21.

288. Warner, J. A., Jones, C. A., Jones, A. C., Miles, E. A., Francis, T. & Warner, J. O. (1997) Immune responses during pregnancy and the development of allergic disease. Pediatric Allergy & Immunology 8: 10.

289. Litonjua, A. A., Carey, V. J., Burge, H. A., Weiss, S. T. & Gold, D. R. (1998) Parental history and the risk for childhood asthma. Does mother confer more risk than father? American Journal of Respiratory & Critical Care Medicine 158: 176-181.

290. Ruiz, R. G., Kemeny, D. M. & Price, J. F. (1992) Higher risk of infantile atopic dermatitis from maternal atopy than from paternal atopy. Clinical & Experimental Allergy 22: 762-766.

291. Prescott, S. L., Macaubas, C., Smallacombe, T., Holt, B. J., Sly, P. D., Loh, R. & Holt, P. G. (1998) Reciprocal age-related patterns of allergen-specific T-cell immunity in normal vs. atopic infants. Clinical & Experimental Allergy 28: S44.

292. Prescott, S. L. & Holt, P. G. (1998) Abnormalities in cord blood mononuclear cytokine production as a predictor of later atopic disease in childhood. Clinical & Experimental Allergy 28: 1313-1316.

293. Kjellman, N. I. (1994) IgE in neonates is not suitable for general allergy risk screening. Pediatric Allergy & Immunology 5: 1-4.

294. Ozol, D. & Mete, E. (2008) Asthma and food allergy. Current Opinion in Pulmonary Medicine 14: 9-12.

295. Komatsu, Y., Fujimoto, K., Yasuo, M., Urushihata, K., Hanaoka, M., Koizumi, T. & Kubo, K. (2009) Airway hyper-responsiveness in young adults with asthma that remitted either during or before adolescence. Respirology 14: 217-223.

296. Anto, J. M., Sunyer, J., Basagana, X., Garcia-Esteban, R., Cerveri, I., de Marco, R., Heinrich, J., Janson, C., Jarvis, D. et al. (2010) Risk factors of new-onset asthma in adults: a population-based international cohort study. Allergy DOI 10.1111/j.1398-9995.2009.02301.x.

297. Kogevinas, M., Zock, J. P., Jarvis, D., Kromhout, H., Lillienberg, L., Plana, E., Radon, K., Toren, K., Alliksoo, A. et al. (2007) Exposure to substances in the workplace and new-onset asthma: an international prospective population-based study (ECRHS-II). Lancet 370: 336-341.

298. Jamrozik, E., Knuiman, M. W., James, A., Divitini, M. & Musk, A. W. (2009) Risk factors for adult-onset asthma: a 14-year longitudinal study. Respirology 14: 814-821.

299. Prescott, S. L. & Dunstan, J. A. (2005) Immune dysregulation in allergic respiratory disease: the role of T regulatory cells. Pulmonary Pharmacology & Therapeutics 18: 217-228.

300. Yang, I. A., Fong, K. M., Holgate, S. T. & Holloway, J. W. (2006) The role of Toll-like receptors and related receptors of the innate immune system in asthma. Current Opinion in Allergy & Clinical Immunology 6: 23-28.

301. Martino, D. J. & Prescott, S. L. (2010) Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease. Allergy 65: 7-15.

302. Noakes, P. S., Thomas, R., Lane, C., Mori, T. A., Barden, A. E., Devadason, S. G. & Prescott, S. L. (2007) Association of maternal smoking with increased infant oxidative stress at 3 months of age. Thorax 62: 714-717.

303. Noakes, P. S., Hale, J., Thomas, R., Lane, C., Devadason, S. G. & Prescott, S. L. (2006) Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses. European Respiratory Journal 28: 721-729.

304. Noakes, P. S., Taylor, P., Wilkinson, S. & Prescott, S. L. (2006) The relationship between persistent organic pollutants in maternal and neonatal tissues and immune responses to allergens: A novel exploratory study. Chemosphere 63: 1304-1311.

305. Hollingsworth, J. W., Maruoka, S., Boon, K., Garantziotis, S., Li, Z., Tomfohr, J., Bailey, N., Potts, E. N., Whitehead, G. et al. (2008) In utero supplementation with methyl donors enhances allergic airway disease in mice. Journal of Clinical Investigation 118: 3462-3469.

306. Haberg, S. E., London, S. J., Stigum, H., Nafstad, P. & Nystad, W. (2009) Folic acid supplements in pregnancy and early childhood respiratory health. Archives of Disease in Childhood 94: 180-184.

307. Jennings, S. & Prescott, S. L. (2010) Early dietary exposures and feeding practices: role in pathogenesis and prevention of allergic disease? Postgraduate medical journal 86: 99.

308. Vance, G. H., Lewis, S. A., Grimshaw, K. E., Wood, P. J., Briggs, R. A., Thornton, C. A. & Warner, J. O. (2005) Exposure of the fetus and infant to hens' egg ovalbumin via the placenta and breast milk in relation to maternal intake of dietary egg. Clinical & Experimental Allergy 35: 1318-1326.

309. Devereux, G., Barker, R. N. & Seaton, A. (2002) Antenatal determinants of neonatal immune responses to allergens. Clinical & Experimental Allergy 32: 43-50.

310. Allan, K., Kelly, F. J. & Devereux, G. (2009) Antioxidants and allergic disease: a case of too little or too much? Clinical & Experimental Allergy 40: 370-380.

311. Wallace, F. A., Neely, S. J., Miles, E. A. & Calder, P. C. (2000) Dietary fats affect macrophage-mediated cytotoxicity towards tumour cells. Immunology and Cell Biology 78: 40-48.

312. Calder, P. C. & Deckelbaum, R. J. (2008) Omega-3 fatty acids: Time to get the messages right! Current Opinion in Clinical Nutrition and Metabolic Care 11: 91-93.

313. Rees, D., Miles, E. A., Banerjee, T., Wells, S. J., Roynette, C. E., Wahle, K. W. & Calder, P. C. (2006) Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. American Journal of Clinical Nutrition 83: 331-342.

314. Yaqoob, P. & Calder, P. (1995) Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. Cellular Immunology 163: 120-128.

315. Kuhnert, M., Strohmeier, R., Stegmuller, M. & Halberstadt, E. (1998) Changes in lymphocyte subsets during normal pregnancy. European Journal of Obstetrics Gynecology and Reproductive Biology 76: 147-151.

316. Mahmoud, F., Abul, H., Omu, A. & Haines, D. (2005) Lymphocyte sub-populations in gestational diabetes. American Journal of Reproductive Immunology 53: 21-29.

317. Mahmoud, F., Abul, H., Omu, A., Al-Rayes, S., Haines, D. & Whaley, K. (2001) Pregnancy-associated changes in peripheral blood lymphocyte subpopulations in normal Kuwaiti women. Gynecologic and Obstetric Investigation 52: 232-236.

318. Zhao, J. X., Zeng, Y. Y. & Liu, Y. (2007) Fetal alloantigen is responsible for the expansion of the CD4+CD25+ regulatory T cell pool during pregnancy. Journal of Reproductive Immunology 75: 71-81.

319. Taams, L., Vukmanovic-Stejic, M., Salmon, M. & Akbar, A. (2003) Immune regulation by CD4+ CD25+ regulatory T cells: Implications for transplantation tolerance. Transplant Immunology 11: 277-285.

320. Tilburgs, T., Roelen, D. L., van der Mast, B. J., de Groot-Swings, G. M., Kleijburg, C., Scherjon, S. A. & Claas, F. H. (2008) Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. Journal of Immunology 180: 5737-5745.

321. Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S. I. et al. (2006) Expression of interleukin (IL)-2 and IL-7 receptors

discriminates between human regulatory and activated T cells. Journal of Experimental Medicine 203: 1693-1700.

322. Sargent, I. L., Borzychowski, A. M. & Redman, C. W. G. (2006) NK cells and human pregnancy - an inflammatory view. Trends in Immunology 27: 399-404.

323. Koga, K., Aldo, P. B. & Mor, G. (2010) Toll-like receptors and pregnancy: trophoblast as modulators of the immune response. Journal of Obstetrics and Gynaecology Research 35: 191-202.

324. Acar, N., Ustunel, I. & Demir, R. (2009) Uterine natural killer (uNK) cells and their missions during pregnancy: A review. Acta Histochemica DOI 10.1016/j.acthis.2009.12.001.

325. Ho, H. N., Chao, K. H., Chen, H. F., Chen, S. U., Wu, M. Y. & Yang, Y. S. (2001) Distribution of Th1 and Th2 cell populations in human peripheral and decidual T cells from normal and anembryonic pregnancies. Fertility & Sterility 76: 797-803.

326. Langer-Gould, A., Gupta, R., Huang, S., Hagan, A., Atkuri, K., Leimpeter, A. D., Albers, K. B., Greenwood, E., Van Den Eeden, S. K. et al. (2010) Interferon-gamma-producing T cells, pregnancy, and postpartum relapses of multiple sclerosis. Archives of Neurology 67: 51-57.

327. Prescott, S. L., Breckler, L. A., Witt, C. S., Smith, L., Dunstan, J. A. & Christiansen, F. T. (2010) Allergic women show reduced T helper type 1 alloresponses to fetal human leucocyte antigen mismatch during pregnancy. Clinical and Experimental Immunology 159: 65-72.

328. Breckler, L. A., Hale, J., Taylor, A., Dunstan, J. A., Thornton, C. A. & Prescott, S. L. (2008) Pregnancy IFN-gamma responses to foetal alloantigens are altered by maternal allergy and gravidity status. Allergy: European Journal of Allergy and Clinical Immunology 63: 1473-1480.

329. Sakai, M., Shiozaki, A., Sasaki, Y., Yoneda, S. & Saito, S. (2004) The ratio of interleukin (IL)-18 to IL-12 secreted by peripheral blood mononuclear cells is increased in normal pregnant subjects and decreased in pre-eclamptic patients. Journal of Reproductive Immunology 61: 133-143.

330. Holmes, V. A., Wallace, J. M., Gilmore, W. S., McFaul, P. & Alexander, H. D. (2003) Plasma levels of the immunomodulatory cytokine interleukin-10 during normal human pregnancy: a longitudinal study. Cytokine 21: 265-269.

331. Trebble, T., Arden, N. K., Stroud, M. A., Wootton, S. A., Burdge, G. C., Miles, E. A., Ballinger, A. B., Thompson, R. L. & Calder, P. C. (2003) Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. British Journal of Nutrition 90: 405-412.

332. Damsgaard, C. T., Lauritzen, L., Calder, P. C., Kjaer, T. R. & Frokiaer, H. (2009) Reduced ex vivo interleukin-6 production by dietary fish oil is not modified by linoleic acid intake in healthy men. Journal of Nutrition 139: 1410-1414.

333. Nagamatsu, T. & Schust, D. J. (2010) Review: the immunomodulatory roles of macrophages at the maternal-fetal interface. Reproductive Sciences 17: 209-218.

APPENDIX

APPENDIX (CD)- Table of contents

Appendix A: 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

Appendix B: 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

Appendix C: SIPS 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…….

Appendix D: SIPS 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

Appendix E: SIPS power calculation

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

Fatty acid composition of plasma phospholipids. Based upon previous work, the content of EPA in plasma phospholipids in these women was expected to be approx. 1% of fatty acids. Two salmon meals per week were anticipated to provide a total of approximately 4 g EPA + DHA/week; of this about 2.8 g will 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 adult women 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 $p < 0.001$).

Sensitisation to at least one common allergen. Infants born to women recruited into the study will be at high risk of becoming sensitized to allergens. Based upon previous studies of such cohorts of infants in Southampton 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 will be 30% less likely to have been sensitized (this is based upon data from Dunstan *et al.* using fish oil in at risk pregnant women). A sample size of 50 per group will have a 70% power to detect this difference with $p < 0.05$.

Maternal body composition. A sample size of 100 subjects was proposed to be sufficient to establish prediction equations at each stage of pregnancy. If unified measurements made at each of three stages of pregnancy are pooled this would involve 300 pregnancy measurements. A sample size of 100 at each time point would be sufficient to establish a correlation as low as 0.26 ($r^2 = 0.07$) between bioelectrical impedance indices and birth weight, with 80% power and $p< 0.05$. The same sample size is sufficient to detect a 7 ohm difference in reactance between the control and intervention group (mean in late pregnancy ~60 + 12 ohms) with $p = 0.05$ and power of 80%.

Appendix F: SIPS 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*

Appendix G: 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…….

Appendix H: 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 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
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 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
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 pints 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	☐

Appendix I: SIPS Salmon consumption diary during pregnancy

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	

Appendix J: SIPS Fish consumption dairy during pregnancy

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	

Appendix K: 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

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

☐

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…….

Appendix L: 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

e) **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…….

Appendix M: Institute of Human Nutrition fatty acid methyl ester standard mix for gas chromatography

Fatty acid methyl ester		**Concentration in mixture (mg/mL)**
Lauric	12:0	1
Myristic	14:0	2
Palmitic	16:0	1
Palmitoleic	16:1n-7	2
Stearic	18:0	1
Oleic	18:1n-9	2
Linoleic	18:2n-6	1
γ-linolenic	18:3n-6	1
α-linolenic	18:3n-3	2
Arachidic	20:0	2
Eicosenoic	20:1n-9	1
Eicosadienoic	20:2n-6	1
Dihomo-γ-linolenic	20:3n-6	1
Arachidonic	20:4n-6	2
Behenic	22:0	2
Lignoceric	24:0	1
Nervonic	24:1n-9	2

Appendix N: Gas chromatography settings for analysis of fatty acid methyl esters using a BPX-70 column

1. Oven

Max temperature		250ºC
Equilibration time		1 min
Initial temperature		115ºC
Initial time		2 min
Ramp 1	Rate	10ºC/min
	Final temperature	200ºC
	Final time	18.5 min
Ramp 2	Rate	60ºC/min
	Final temperature	245ºC
	Final time	8 min
Run time	37.75 min	

2. Front inlet

Temperature	300ºC
Pressure	14.75psi
Gas saver	on
Saver flow	20mL/min
Saver time	2 min
Gas type	Helium

3. Column

Length	30m
Diameter	220µm
Film thickness	0.25µm
Initial flow	0.1mL/min
Average velocity	31cm/min
Outlet pressure	ambient

4. Detector

Temperature	300ºC
Hydrogen flow	40mL/min
Air flow	184mL/min
Constant makeup flow	
Makeup flow	45L/min
Makeup gas	Helium
Electrometer	on
Lit offset	2