UNIVERSITY OF SOUTHAMPTON

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Effect of Fatty Acids and Programming on the Immune System

by

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ABSTRACT

DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE, SCHOOL OF MEDICINE

Doctor of Philosophy

EFFECT OF FATTY ACIDS AND PROGRAMMING ON THE IMMUNE SYSTEM

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Research to date has suggested that fatty acids (FAs) may affect the immune system, through their (and those of their metabolites) effects on membranes, mediators, and gene expression. However, despite the research carried out, there still exist gaps of knowledge where further research is required. In addition, programming by diet in pregnancy may affect the immune system, due to stress and/or structural and functional changes to immune cells, but whether this effect is long-lasting is uncertain. In order to address some of these gaps in knowledge, experiments were conducted to examine the effects of dietary FAs on immune outcomes in rodent models of clinical relevance and the effect of maternal protein restriction on immune outcomes in two later generations.

The first experiment investigated the effect of diets differing in FA composition on the recall response in a mouse model of influenza vaccination. A diet rich in salmon oil resulted in enhanced ear swelling (measured after 48 hours) compared with diets rich in linseed oil, sunflower oil or beef tallow, suggesting that long chain *n-3* FAs increase the T_H1 response. The different effects of salmon oil compared with the other diets appears to relate to the lower *n-6* fatty acid status that occurs with salmon oil feeding. The second experiment investigated the effect of diets differing in FA composition in a mouse model of allergic sensitisation (to ovalbumin). There was reduced immediate hypersensitivity to ovalbumin in mice fed a diet rich in salmon oil and increased immediate hypersensitivity in mice fed a diet rich in beef tallow, but there was no effect of diet on airway responsiveness to ovalbumin. Beef tallow feeding also raised IgE in blood and elevated IL-4 production by anti-CD3 stimulated splenocytes. The salmon oil did not affect IgE or cytokine profiles. The results suggest that the *n-3* FAs found in salmon oil decrease T_H2-mediated responses to an allergen and that such responses are increased by a diet rich in saturated FAs. The third experiment examined the effect of diets differing in FA composition fed during rat pregnancy on the abundance of different immune cells in blood and lymphoid organs of the offspring at weaning and beyond. The FA profile of the dams at the end of lactation reflected dietary intake in pregnancy, but no changes were found in immune cell abundance of offspring. The fourth experiment examined the effect of diets rich in α-linolenic acid (ALA, a short chain *n-3* FA) or linoleic acid (LA, a short chain *n-6* FA) in mouse pregnancy on allergic sensitisation to ovalbumin in the offspring. Diets were given during pregnancy alone, in both gestation and lactation, or during lactation alone, and compared to a low-fat control. The ALA-rich diet in gestation and lactation and the LA-rich diet in gestation reduced ear swelling in the offspring. Despite these differences, there were no effects on lung function or immune markers. The fifth experiment investigated the effect of protein restriction in rat pregnancy on the immune system of the offspring over two generations. No differences were seen between groups in the F1 generation. Protein restriction reduced T_H and B-cell abundance in the spleen and thymus of F2 males.

In conclusion, dietary fatty acids can alter immune responses, and there may be an effect of FAs in pregnancy on the immune response in the offspring but such effects may not be long lasting. Protein restriction during pregnancy, which is stress inducing, appears to have an effect on immune cell abundance that is manifested at the second generation of offspring.

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DECLARATION OF AUTHORSHIP

I, Alison Lindsay Fear

declare that the thesis entitled

Effect of Fatty Acids and Programming on the Immune System

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;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have 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;
- parts of this work have been published as:

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Abbreviations

The following abbreviations were used in this thesis.

AA	Arachidonic acid, 20:4 *n-6*
ALA	α-Linolenic acid, 18:3 *n-3*
AG	(Dietary group in Chapter 5) received ALA-rich diet in gestation
AGL	(Dietary group in Chapter 5) received ALA-rich diet in gestation and lactation
APC	Antigen-presenting cell
BALF	Bronchiolar lavage fluid
BAT	Brown adipose tissue
BSA	Bovine serum albumin
C	(Dietary group in Chapter 6) F0 and F1 control (18 % protein)
CD	Cluster of differentiation
CE	Cholesteryl ester
CF	(Dietary group in Chapter 6) F1 control + folate
CLA	Conjugated linoleic acid
Con A	Concanavalin A
COX	Cyclo-oxygenase
CTL	Cytotoxic T-lymphocyte
DAG	Diacylglycerol
DGLA	Dihomo-γ-linoleic acid, 20:3 *n-6*
DHA	Docosaxaenoic acid, 22:6 *n-3*
DTH	Delayed-type hypersensitivity
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid, 20:5 *n-3*
EPO	Evening primrose oil; rich in GLA
FA	Fatty acid
FABP	Fatty acid binding protein
FACs	Flow cytometry
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FO	Fish oil; rich in long chain *n-3* PUFAs
FSC	Forward scatter

GC	Gas chromatography
GLA	γ-Linolenic acid, 18:3 *n-6*
H	Haemagglutinin
HCO	Hydrogenated coconut oil; rich in SFAs
HDL	High-density-lipoprotein
HETE	Hydroxyeicosatetraenoic acid
HSL	Hormone-sensitive lipase
IDL	Intermediate-density-lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IH	Immediate hypersensitivity
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LA	Linoleic acid, 18:2 *n-6*
LDL	Low-density-lipoprotein
LG	(Dietary group in Chapter 5) received LA-rich diet in gestation
LGL	(Dietary group in Chapter 5) received LA-rich diet in gestation and lactation
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatability complex
MUFA	Monounsaturated fatty acid
N	Neuraminidase
NEFA	Non-esterified fatty acid
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	Oleic acid, 18:1 *n-9*
OO	Olive oil; rich in OA
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PG	Prostaglandin
PKC	Protein kinase C
PL	Phospholipid

PPAR	Peroxisome proliferation-activated receptor
PR	(Dietary group in Chapter 6) F0 protein restricted (9 %)
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPE	r-phycoerythrein
SAFO	Safflower oil, rich in linoleic acid 18:2 *n-6*
SCD	Stearoyl CoA desaturase, Δ-9 desaturase
SFA	Saturated fatty acid
SLE	Systemic lupus erythematosus
sn	Stereospecific numbering
SSC	Side scatter
TAG	Triacylglycerol
T_H	T-helper cell
TNF	Tumour necrosis factor
T_{REGS}	Regulatory T-cells
TX	Thromboxane
UCP1	Uncoupling protein 1
VLDL	Very-low-density-lipoprotein
WAT	White adipose tissue

Chapter 1. Literature Review

1.1. Introduction

The experiments described in this thesis use rat and mouse models to examine the effect of dietary fatty acids and diet in pregnancy on aspects of the immune system. The literature review therefore provides background information on the immune system, diet in pregnancy and fatty acids. Previous studies investigating the effect of fatty acids and diet in pregnancy on the immune system will also be discussed, along with their limitations, and areas requiring further research.

1.2. The Immune System

The word “immunity” is derived from the Latin word “immunis” meaning exempt from service or liability, with the word “immune” meaning being secure from a contagion [1]. This state is achieved by the actions of the immune system, which acts to defend the body from pathogenic bacteria, viruses and fungi, functioning by the complex interaction of various cells and molecules.

1.2.1. Cells of the Immune System

The main cells of the immune system are lymphocytes (T-, B- and NK cells), dendritic cells, mononuclear phagocytes (monocytes and macrophages) and granulocytes (basophils, eosinophils and neutrophils), which are collectively known as leukocytes (or white blood cells when in the bloodstream). The relative proportions of these cells in peripheral blood of humans, rats and mice are shown in Table 1-1. Humans appear to produce relatively more cells of the innate immune system than mice or rats. Whilst this may mean an increase in difficulty in translating research from this thesis which uses rat and mice models, to the situation occurring in humans, animal models provide several benefits making them suitable for research, such as reduced gestation time, ease of control of diet and environmental factors, ease of availability of tissue samples etc. In addition, data from this thesis focuses on the adaptive immune response. However, differences between the human immune system cell abundance, and that of the mice and rat should be noted.

Table 1-1: Different leukocytes (as a proportion of total leukocytes) in the blood of humans and rats. Human data are taken from Harrison [2], and rat and mouse data taken from Wolfenson and Lloyd [3].

Leukocytes	Human	Rat	Mouse
Neutrophils	45 - 74	9 - 34	10 - 40
Lymphocytes	16 - 45	65 - 85	55 – 95
Eosinophils	0 - 7	0 - 6	0 – 4
Monocytes	4 - 10	0 - 5	0.1 – 0.35
Basophils	0 - 2	0 - 1.5	0 – 0.3

All blood cells originate from the same progenitors, haematopoietic stem cells in bone marrow, and their development is regulated by cytokines and related growth factors (Figure 1-1). Cytokines produced during immune responses can also regulate the differentiation of these stem cells, altering the balance of the types of cells produced.

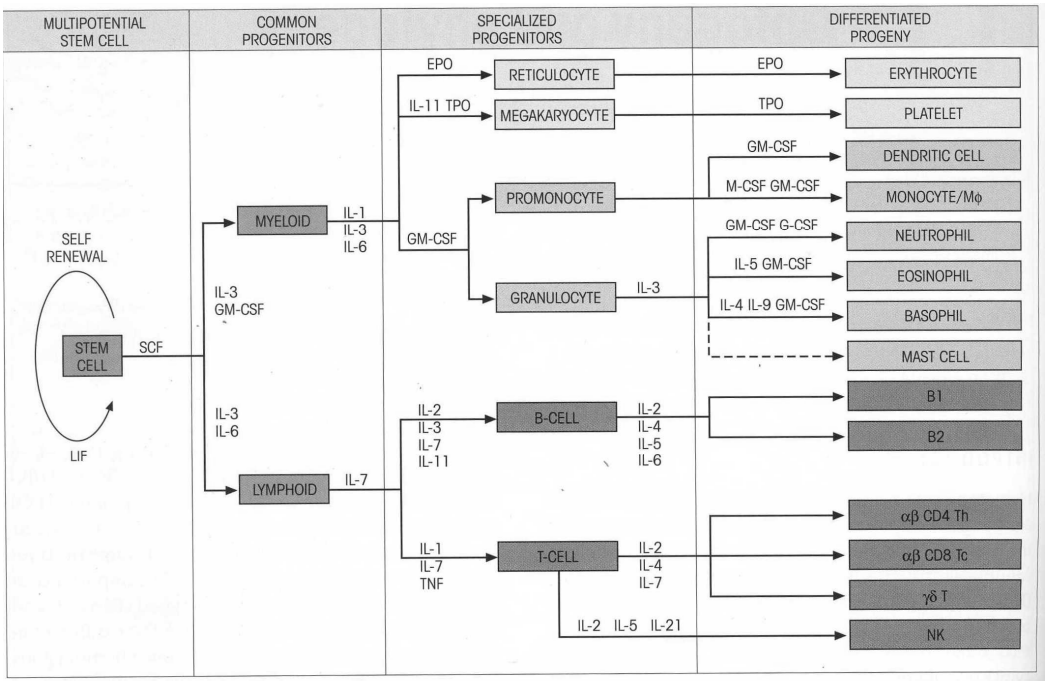

Figure 1-1: Haematopoiesis. The stem cell differentiates in the bone marrow under the influence of various soluble growth factors. SCF, stem cell factor; LIF, leukaemia inhibitory factor; IL-3, interleuki*n-3*, multi-CSF; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, monocyte colony-stimulating factor; EPO, erythropoietin; TPO, thrombopoetin; TNF, tumour necrosis factor. Taken from Delves *et al.* [4].

1.2.1.1. Lymphocytes

There are three classes of lymphocytes – B-cells, T-cells and NK cells. B- and T-cells function in adaptive immunity, whilst NK cells are involved in innate immunity. T-cells can be further subdivided into T helper (T_H) cells, cytotoxic T-cells (CTLs), and regulatory T-cells (T_{REGS}), depending on the CD markers they express. CD phenotype and function of these cells are detailed in Table 1-2.

Table 1-2: Types of T-cell, CD markers expressed, and function.

T-cell	CD markers	Function
T helper (T_H) cells	$CD3^+CD4^+CD8^-$	Stimulate B-cell growth and differentiation (humoral immunity, see 1.2.3.2.1) Activate macrophages (cell-mediated immunity, see 1.2.3.2.2)
Cytotoxic T-cells (CTLs)	$CD3^+CD4^-CD8^+$	Kill virus-infected cells, tumour cells, rejection of allografts (cell-mediated immunity, see 1.2.3.2.2)
Regulatory T-cells (T_{REGS})	$CD3^+CD4^+CD25^+$ FoxP3	Suppress activation of the immune system, maintaining tolerance to self-antigens Regulates development and function of T_{REGS}

Although lymphocytes express different proteins, such as CD markers and antigen receptors, they are morphologically indistinguishable.

B-cells remain in bone marrow while they mature, whilst T-cells mature in the thymus. Maturation involves a series of sequential steps, causing changes in the expression of different genes and surface markers, and acquisition of functional capabilities. This is summarised in Figure 1-2.

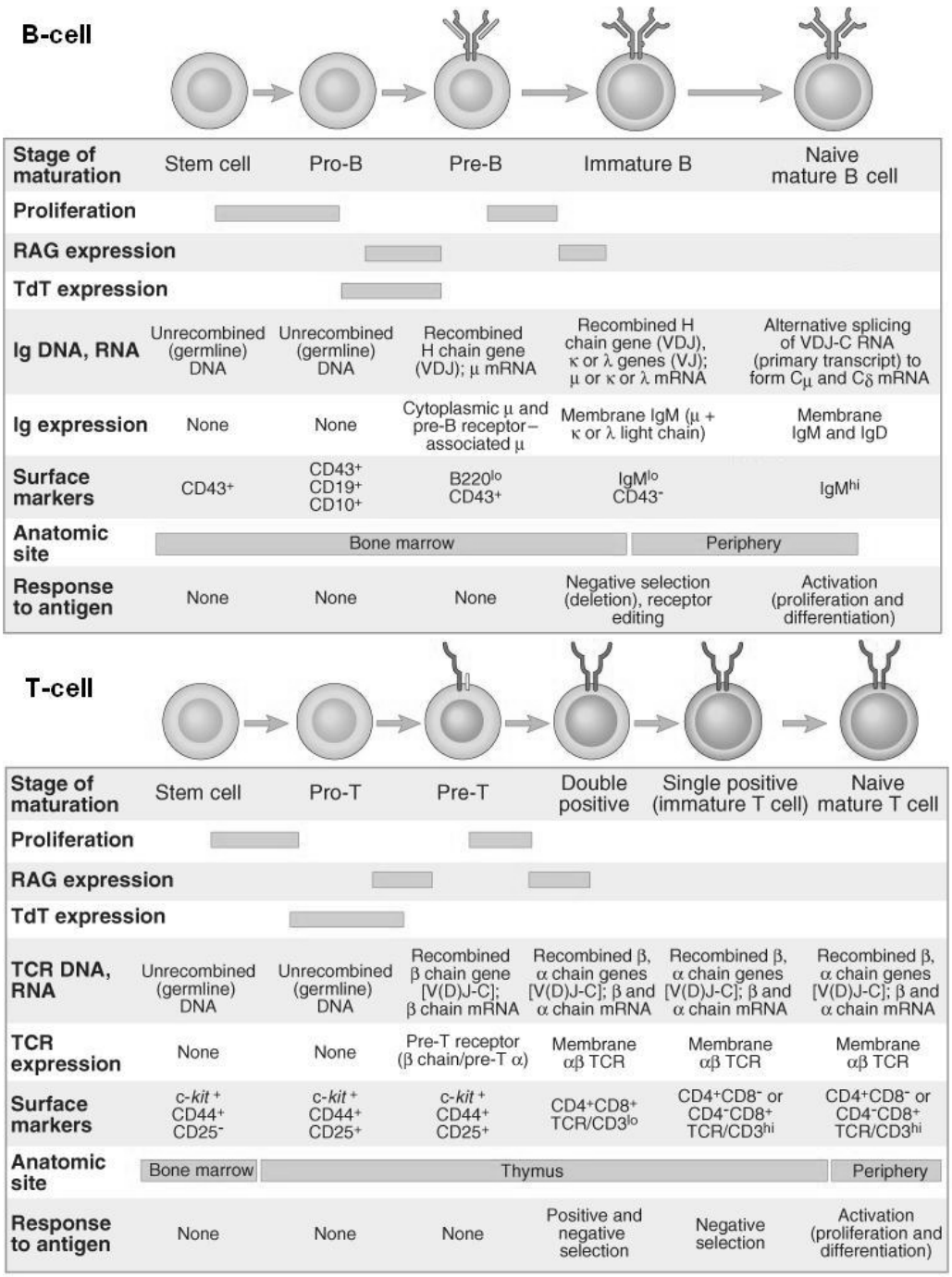

Figure 1-2: Steps in the maturation of B- (top) and T-cells (bottom). TdT, terminal deoxyribonucleotidyl transferase. Figure adapted from Abbas and Lichtman [5].

NK cells function in innate immunity to kill infected cells, and cells that do not express class I MHC molecules. They are phenotypically distinct from T- and B-cells; human NK cells are $CD56^+$ $CD3^-$ lymphocytes [6]. Whilst development of T- and B-cells has been extensively studied, NK cell development is less well understood [4, 7]. Current thinking suggests that NK cells may mature in the thymus and bone marrow, giving rise to distinct phenotypes and functional capabilities depending on the site of development [8].

Prior to antigen stimulation, lymphocytes are termed naïve. They are approximately 8 to 10 µm in diameter [5], and are in the G_0 stage of the cell cycle. Following stimulation, lymphocytes enter the G_1 phase of the cell cycle, where they transcribe genes that were previously silent, synthesize new proteins, grow to 10 to 12 µm in diameter, and are known as large lymphocytes or lymphoblasts [5].

Lymphocytes then undergo mitosis, resulting in rapid proliferation, known as clonal expansion, and differentiate into effector cells or memory cells. Effector cells (CTLs, T_H cells, antibody-secreting B-cells) act to remove the antigen, whilst memory cells allow a faster and greater response to subsequent exposure to the same antigen. Memory cells express surface markers that distinguish them from naïve mature T- and B-cells, and effector cells, and can exist in the body for many years following after the original exposure to the antigen. More detail on the activities of effector cells can be found in section 1.2.3.2.

1.2.1.2. Antigen-Presenting Cells

Antigen-presenting cells (APCs) display captured antigens to lymphocytes, and provide the signals necessary for lymphocyte activation. All nucleated cells can present cytosolic antigens to CTLs, using class I MHC molecules. Specialised APCs, such as dendritic cells, macrophages and B-cells present extracellular antigens to T_H cells using class II MHC molecules. This is summarised in Figure 1-3.

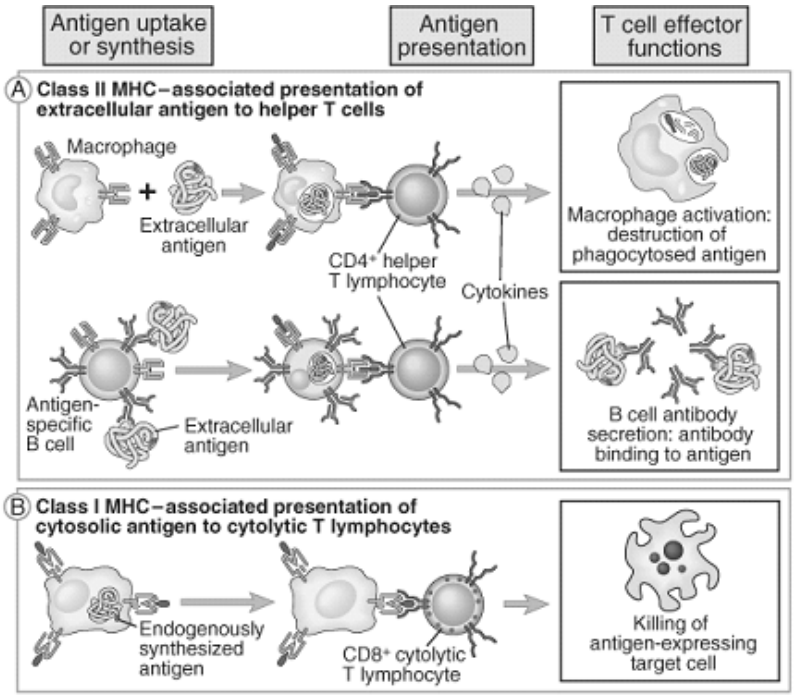

Figure 1-3: Presentation of antigens by antigen presenting cells. Picture A shows the presentation of an extracellular antigen to T_H cells, which results in macrophage or B cell activation and elimination of the antigen. Picture B shows the presentation of a cytosolic antigen to a CTL, and lysis of the antigen-expressing target cell. Picture taken from Abbas and Lichtman [5].

Dendritic cells are found in lymphoid organs, skin epithelia, gastrointestinal and respiratory tracts, and most parenchymal organs. They are easily identifiable by their membranous projections. Monocytes originate in bone marrow, and are 10 to 15 μm in diameter. They mature into macrophages when they settle in tissues, and can be activated by external stimuli, such as microbes, into different morphological forms. Macrophages are present in all organs and connective tissues, and are also involved in innate immunity. Figure 1-4 shows dendritic cells, a monocyte and a macrophage.

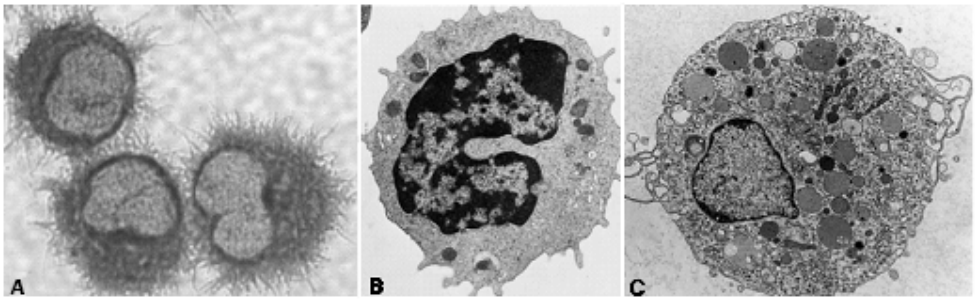

Figure 1-4: Antigen presenting cells. Picture A shows a light micrograph of cultured dendritic cells. Figure B shows an electron micrograph of a peripheral blood monocyte. Figure C shows an electron micrograph of an activated tissue macrophage. Adapted from Abbas and Lichtman [5].

1.2.1.3. Mast cells

Mast cells, along with basophils and eosinophils are the effector cells of immediate hypersensitivity reactions and allergy (see 1.2.4). Mast cell progenitors migrate to peripheral tissues where they differentiate into mature cells. Cells vary in shape, have round nuclei, and membrane-bound granules and lipid bodies in the cytoplasm. In the granules are acidic proteoglycans, which bind basic dyes. An electron micrograph of a mast cell is shown in Figure 1-5. Mast cells induce the acute inflammatory reaction of innate immunity.

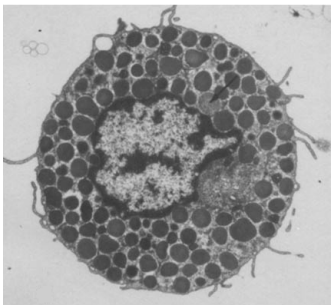

Figure 1-5: An electron micrograph of a resting mast cell, containing membrane bound granules. Taken from Lawson *et al.* [9].

1.2.1.4. Granulocytes

Granulocytes contain granules in their cytoplasm and can be sub-classified into neutrophils, eosinophils and basophils depending on the colour and morphology of the granules when stained with Romanowsky stains (Figure 1-6).

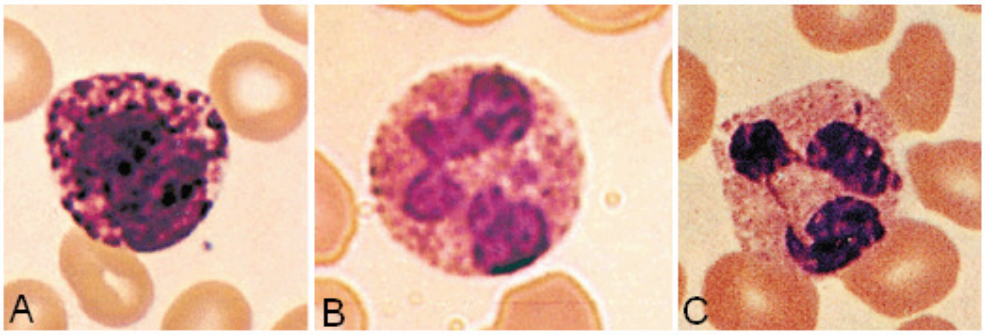

Figure 1-6: Granulocytes. Picture A shows a human peripheral blood basophil stained with Wright. Picture B shows an eosinophil stained with May-Grünwald-Giemsa. Picture C shows a neutrophil stained with Wright. Figure adapted from figures 12.2A, 11.1, 10.9A in Wintrobe's Clinical Hematology [10].

Basophils are not usually present in tissues, but circulate in the blood, from where they can be recruited to sites of infection. They produce histamine and heparin, like mast cells, and are involved in the inflammation reactions of both immediate and late-phase reactions of immediate hypersensitivity.

Eosinophils circulate in the blood after maturation in the bone marrow. They are common in peripheral tissues, but their abundance increases at sites of late-phase inflammation. Activated eosinophils, along with basophils and mast cells, release lipid mediators, including platelet activating factor (PAF), prostaglandins and leukotrienes, which are involved in the pathological processes of allergy. Eosinophils are also responsible for the killing of helminthic parasites.

Neutrophils are the most abundant circulating leukocyte and mediate the earliest stages of the inflammatory response. Neutrophils circulate in the blood for about six hours unless they are recruited to sites of infection. If there is no infection, they undergo programmed cell death.

1.2.2. Tissues of the Immune System

The cells of the immune system are present circulating the blood and lymphatic system, in anatomically defined lymphoid organs (bone marrow, thymus, spleen and lymph nodes) and as scattered cells in almost all tissues. The lymphoid organs and lymphatic system are shown in Figure 1-7.

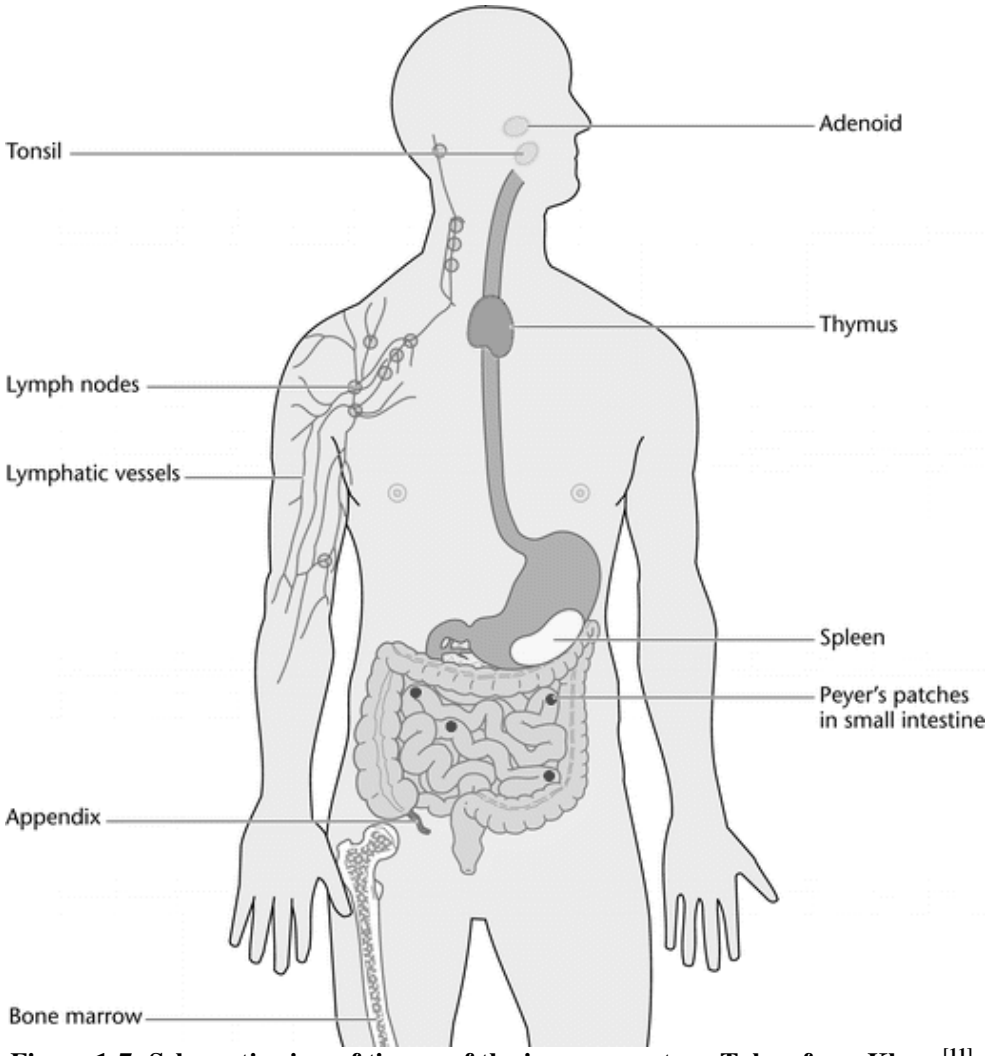

Figure 1-7: Schematic view of tissues of the immune system. Taken from Klaus [11].

1.2.2.1. Bone Marrow

The bone marrow is the site of generation of all adult blood cells (haematopoiesis), and maturation of these cells except T-cells and some NK cells (see 1.2.1.1). In embryonic and early life, haematopoiesis occurs in other sites, such as the blood islands of the yolk sac, the para-aortic mesochyme, the liver and the spleen. Haematopoiesis occurs in the red marrow, whilst yellow marrow contains fat-filled cells; approximately 4×10^{11} cells are produced in the bone marrow of adults every day [12]. Blood cell precursors mature and leave the bone marrow, entering the vascular circulation.

1.2.2.2. Thymus

The thymus is a bi-lobed organ situated above and in front of the heart, and is the site of maturation of T-cells and some NK cells. Each lobe is further divided into smaller lobes, which consist of a medulla and cortex. The cortex is densely packed with T-cells,

whilst the medulla contains fewer. The thymus also contains non-lymphoid epithelial cells, bone marrow-derived macrophages, dendritic cells, and has a rich blood supply. The medulla has structures known as Hassall's corpuscles, which are made up of whorls of keratinised epithelial cells. Hassall's corpuscles act to dispose of degenerating thymocytes (i.e. lymphocytes in the thymus). A micrograph of a thymus is shown in Figure 1-8. The thymus increases in size up until puberty, after which it progressively shrinks (involutes).

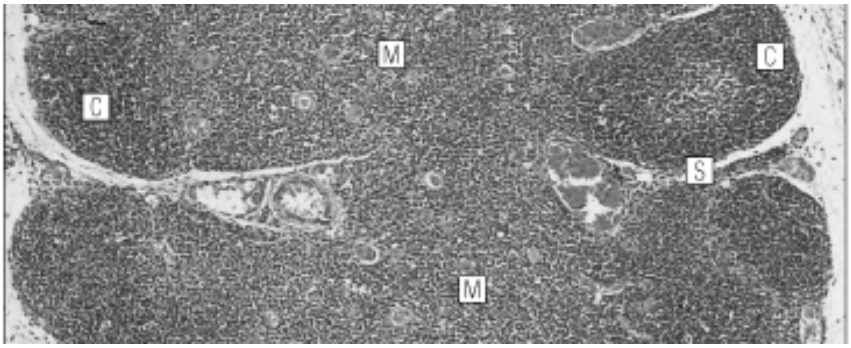

Figure 1-8: Micrograph of the thymus of a child (which is not involuted unlike that of an adult). C, cortex; M, medulla; S, septa. Taken from Stevens and Lowe [13].

1.2.2.3. Spleen

The spleen is located on the left hand side of the body, below and behind the stomach, and is the major site of immune responses to blood-borne antigens. It is supplied by a single splenic artery, which enters the capsule at the hilum, and subsequently divides into smaller branches. The spleen contains both T- and B-cells, in segregated areas, known as periarteriolar lymphoid sheaths and lymphoid follicles respectively. The latter are edged by an area called the marginal zone, which contains lymphocytes and macrophages. These areas make up the white pulp of the spleen, whilst the red pulp is made up of erythrocytes, macrophages, sparse lymphocytes and plasma cells. A micrograph of the spleen is shown in Figure 1-9.

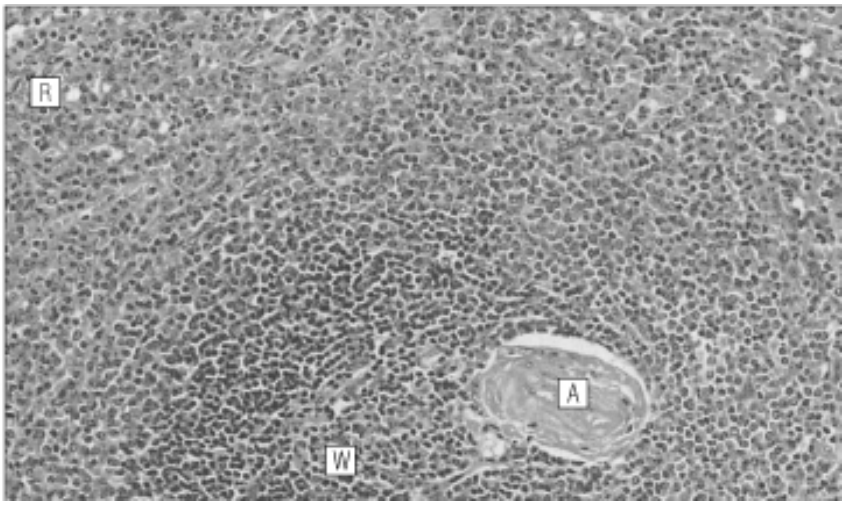

Figure 1-9: Micrograph of the spleen. A, central artery; R, red pulp; W, white pulp. Taken from Stevens and Lowe [13].

1.2.2.4. Lymph Nodes

Adaptive immune responses to lymph-borne antigens are instigated in lymph nodes; these are small nodular masses found on the lymphatic system (a vast network of channels that extends throughout the body). As with the spleen, lymph nodes consist of a medulla and cortex, and T- and B-cells are segregated into different areas (parafollicular cortex and lymphoid follicle respectively). T- and B-cells enter the lymph node through the artery, and are attracted to their respective sections by chemokines; CCR7 attracts T-cells and CXCR5 attracts B-cells.

Lymph is the fluid of the lymphatic system which has previously drained body tissues. By being filtered though lymph nodes, foreign antigens can be displayed to T-cells and induce an immune response. Lymph enters through afferent lymphatic vessels, drains into the cortex and medullary sinus, and leaves through the efferent lymphatic vessel in the hilum.

In order to mount an immune response, lymphocytes must move continuously through the body via the blood stream and lymphatic system (lymphocyte recirculation). Particular kinds of lymphocytes may be attracted to certain regions, whilst others are not, due to a process known as lymphocyte homing. This is achieved predominately by means of adhesion molecules on lymphocytes, endothelial cells and the extracellular matrix, as well as the presence of chemokines produced in the endothelium and tissues.

Naïve T-cells leave the blood and enter lymph nodes through high endothelial venules. If they encounter an antigen, they become activated and return to the circulation through efferent lymphatic vessels, and the thoracic duct, which ultimately allows arterial circulation. From here, they pass to the peripheral sites of infection or inflammation

(through the action of E- and P-selectins and integrins) where they act to eliminate pathogens. If naïve T-cells in lymph nodes do not encounter an antigen, they re-enter circulation through an efferent lymphatic vessel and continue on to other lymph nodes.

1.2.3. The Immune Response

There are two functional components of the immune system, known as innate and adaptive immunity.

1.2.3.1. Innate Immunity

Innate immunity describes the parts of the system which only recognise microbial pathogens, but respond rapidly. It has no memory (a subsequent immunological challenge will not result in a faster and more intense response), and cells do not respond to individual types of microbes or molecules, but rather to the more generic structures (termed "patterns") shared by many different microbes, and which are required for their survival. Innate immunity is achieved through the actions of physical and chemical barriers (epithelial cells and their secretions), cells (macrophages, neutrophils and NK cells), and complement proteins in the blood.

Epithelial surfaces form physical barriers between the external environment and the body, thus preventing microbes from entering, provided they remain intact. They also produce peptides with antibiotic activity, such as defensins, whose synthesis is increased in response to inflammatory cytokines such as interleukin (IL)-1 and tumour necrosis factor (TNF)-α. These are produced by macrophages and other cells (including epithelia), as a result of the presence of microbes in the body. Epithelial sites house intraepithelial T-lymphocytes (in barrier epithelia) and the B-1 subset of B-cells (in serosal cavaties). Macrophages, neutrophils, mast cells and NK cells act when microbes have penetrated the epithelial barrier into tissues or circulation. Macrophages and neutrophils act to phagocytose microorganisms, whilst NK cells release cytotoxic granule proteins to cause apoptosis of target cells. In addition, macrophages produce IL-12, which stimulates NK cells and T-cells to produce interferon (IFN)-γ. This in turn activates macrophages to destroy phagocytosed microorganisms, promotes the production of T_H1 cells and induces cell-mediated immune responses (described further in 1.2.3.2.2).

The complement system consists of plasma proteins that are activated by microorganisms and cause their destruction, as well as inflammation. There are three separate components termed the classical pathway, the alternative pathway and the lectin

pathway. In the classical pathway, C1 (a plasma protein) detects immunoglobulin (Ig) M, IgG1 or IgG3 antibodies on the surface of microorganisms. In the alternative pathway, certain microbial cell surface structures are recognised. The lectin pathway uses mannose-binding lectin (a plasma protein) to recognise and bind to microbial glycoproteins and glycolipids. If any of these pathways is triggered, there is sequential recruitment and conversion of complement proteins into protease complexes. The resulting proteins each have different effector functions; C3b regulates the late steps of complement activation, resulting in the production of peptides that stimulate inflammation (C5a), and polymerise C9, which forms the membrane attack complex. The membrane attack complex is responsible for creating holes in plasma membranes and subsequent lysis of the microorganism. The end result is death of the microbe and acute inflammation (through mast cell activation). In addition, production of the complement protein C3d is the second signal required for B-cell activation (the first is antigen) thereby inducing humoral immune responses. Complement activation is inhibited by regulatory proteins present on normal host cells, but absent from microbes, thereby preventing injury to host cells.

1.2.3.2. Adaptive Immunity

Adaptive immunity is stimulated by exposure to both microbial and non-microbial pathogens. Immune cells recognise antigens present on pathogens, and this triggers a specific immune response. Somatic recombination of gene sections allows the production of a vast array of receptors on immune cells, and although one cell will only recognise one antigen, the sheer number of cells in the immune system allows recognition of, and response to, an enormous number and variety of antigens. It has been estimated that humans can recognise 10^{11} different antigens [14]. Repeated exposure to an antigen-bearing pathogen will result in a faster and more magnified response, described as memory. There are two types of adaptive immunity, known as humoral- and cell-mediated immunity.

1.2.3.2.1. Humoral-mediated Immunity

Humoral-mediated immunity operates through B-cells and the antibodies they produce, which act on extracellular microbes. Each B-cell expresses a set of antibodies on its surface specific for one particular antigen. Binding of the antigen to the antibody causes clonal expansion, and subsequent maturation of B-cells into plasma cells (which secrete antibodies), or memory cells (longer lasting cells that are responsible for the memory of adaptive immunity). T-helper cells are required for the proliferation and differentiation of B-cells. In response to CD40 binding and cytokines, B-cells in peripheral lymphoid tissues

can undergo heavy chain isotype switching, resulting in the production of antibodies with different types of heavy chains, such as γ, α and ε. These antibodies have different effector functions. Isotype switching and antibody functions are shown in Figure 1-10.

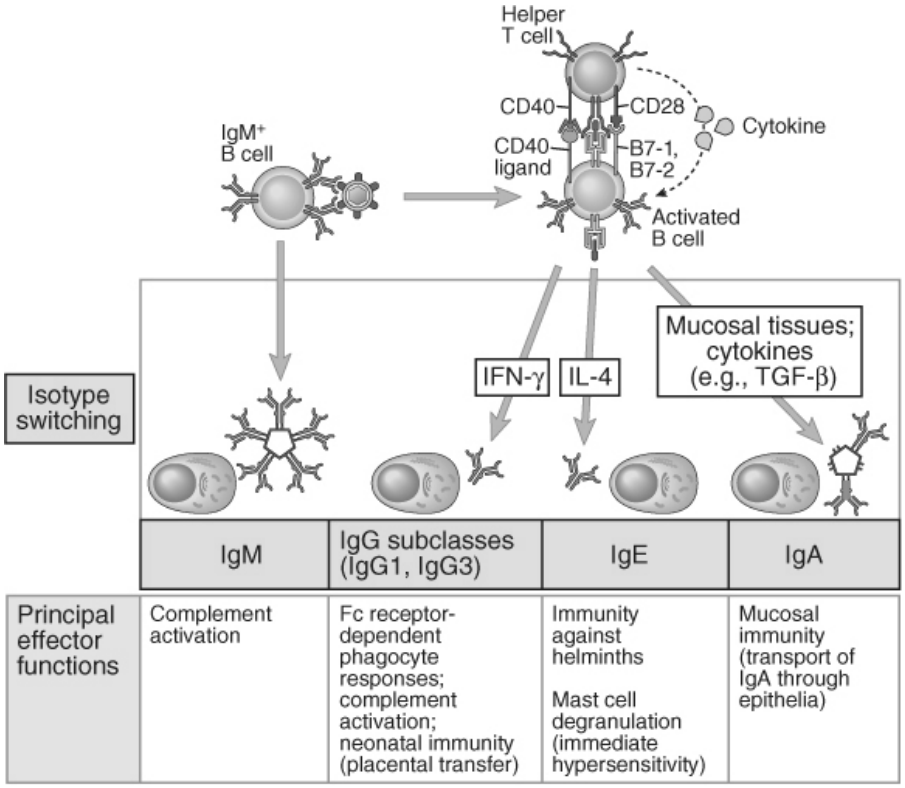

Figure 1-10: Isotype switching. In response to CD40 binding and certain cytokines, B-cells can produce progeny with different antibody isotypes. Taken from Abbas and Lichtman [5].

Antibodies act through several effector mechanisms. Binding of microbes to B-cells results in the production of antibodies, which neutralise microbes and their toxins by opsonising them and promoting their uptake by phagocytosis, causes antibody-dependent cellular cytotoxicity, and complement activation. This leads to lysis of microbes, phagocytosis of microbes that are opsonised with complement fragments, and inflammation.

1.2.3.2.2. Cell-mediated Immunity

Cell-mediated immunity is a result of the activities of T-cells, which target intracellular microbes that exist within host cells, where they are inaccessible to antibodies. There are four main types of T-cells, which can be identified by the proteins expressed on their cell surface, known as “cluster of differentiation” or CD, proteins. T-cells that are $CD4^+CD8^-$ are T-helper (T_H) cells, and can be further subdivided into T_H1 and T_H2 cells. $CD4^-CD8^+$ T-cells are cytotoxic T-cells (CTLs), and are responsible for the killing of virus-infected cells. T_H1 cells recognise class II MHC-associated peptide antigens

presented by antigen presenting cells, while CTLs recognise class I MHC-associated peptide antigens expressed by phagocytes with ingested microbes. Both T-cells secrete cytokines that cause macrophages to kill ingested microbes, and stimulate inflammation. Alternatively, CTLs recognise class I MHC-associated peptide antigens of microbes present in the cytoplasm of infected cells, and kill them.

T_H2 cells secrete the cytokines IL-4, IL-5 and IL-10; IL-4 stimulates the production of antibodies by B-cells, and acts to promote the proliferation and differentiation of T_H2 cells from naïve T-helper cells. IL-5 acts to activate eosinophils to destroy helminthic parasites, whilst IL-10 and IL-4 inhibit macrophage activation. T_H2 cells are also responsible for immediate hypersensitivity reactions (see 1.2.4).

There are several different factors which interact to determine whether T-helper cells are of the T_H1 or T_H2 phenotype. The cytokine IFN-γ stimulates the production of T_H1 cells, whilst IL-4 and IL-10 inhibit T_H1 production, and stimulate T_H2 proliferation. The cytokines that stimulate T_H1 and T_H2 proliferation are also produced by that type of cell, with the result that the T-helper cell phenotype becomes increasingly polarised in one direction. Factors that alter the balance of these cytokines, therefore also have the potential to alter the ability of the host to mount an effective cell-mediated immune response and to alter the likelihood of development of allergy (explored further in 1.4).

Regulatory T-cells are $CD4^+$ with the majority also expressing CD25 [15], and are responsible for the down-regulation of self-reactive T-cells (reviewed by Wing *et al.* [16]). Impaired activity of regulatory T-cells can lead to autoimmune disease and atopy [17]. The final type of T-cell is $CD4^-CD8^-$, and known as a γδT-cell; their function still remains unclear [5].

1.2.4. Hypersensitivity Reactions and Disease

The immune system is not flawless; inappropriate immune responses can occur that lead to tissue damage. Reactions are due to normal immune responses occurring, but in a way that is excessive or improper, and results in tissue injury. Such reactions are known as hypersensitivity reactions and there are four types, classified by the type of immune response and effector mechanisms responsible.

Immediate (Type I) hypersensitivity (IH) reactions (allergy or atopy) occur when an immune response arises as a result of exposure to a protein, or chemical bound to a protein (an allergen), and are caused by T_H2 cells. They occur rapidly (within minutes) following exposure to the antigen [5]. Recognition of an allergen can lead T_H2 cells to change production of antibodies in B-cells to the IgE type. IgE is secreted and binds to

mast cells. Repeated exposure to the allergen causes mast cell activation, and subsequent release of mediators responsible for the clinical signs of allergy (e.g. histamine, leukotrienes).

There are two phases to IH, an immediate and late-phase. Vascular and smooth muscle responses to mediators are characteristic of the immediate reaction, whereas in the late-phase reaction, leukocyte recruitment and inflammation occur. Reactions are manifest in several ways, including skin and mucosal allergies, food allergies and systemic anaphylaxis. Asthma is caused by repeated immediate and late-phase reactions in the lung. This results in intermittent and reversible obstruction of the airway, chronic bronchial inflammation with eosinophils, hypertrophy of bronchial smooth muscle, and hyper-reactivity to bronchoconstrictors (e.g. LTC4, LTD4, LTE4 and PAF). Allergic rhinitis is an IH reaction to allergens such as plant pollen or house dust mites in the upper respiratory tract. This results in mucosal oedema, leukocyte and eosinophil accumulation, mucus secretion, coughing, sneezing, and difficulty breathing. Food allergies can cause increased peristalsis, increased fluid secretion from intestinal epithelia, vomiting and diarrhoea. Utricaria and eczema are characteristic of IH reactions in the skin.

Type II hypersensitivity is mediated by antibodies other than IgE. It occurs when antibodies bind to antigens on certain cells. As a result, inflammatory cells are recruited and activated, and normal cell function is hindered, which induces tissue damage. Examples of diseases caused by this type of hypersensitivity include Goodpasture's syndrome, acute rheumatic fever, Myasthenia gravis, Graves' disease, insulin-resistant (Type II) diabetes, and pernicious anaemia [5].

Type III hypersensitivity occurs when antigen-antibody complexes form in the circulation and are deposited in vessel walls. As with type II hypersensitivity, inflammatory cells are recruited and activated, and cell function hindered, resulting in tissue injury. Examples of diseases that result from this include systemic lupus erythematosus, polyarteritis nodosa, poststreptococcal glomerulonephritis and serum sickness [5]. Whilst production of antibody-antigen complexes occurs during normal immune responses, it is only when they are produced at excessive levels or not removed efficiently that they become deposited in tissues and cause disease.

Type IV hypersensitivity is caused by T cells that either trigger delayed-type hypersensitivity (DTH) or directly kill target cells. This response occurs several hours after antigen exposure, and reaches its maximum at 24 – 48 hours, before subsiding [4]. In DTH, T_H1 cells (and sometimes CTLs) respond to tissue antigens by secreting cytokines that cause inflammation and activate phagocytes, resulting in tissue injury. Examples of

pathological conditions caused by DTH include insulin-dependent (Type I) diabetes, multiple sclerosis, rheumatoid arthritis, sarcoidosis and contact dermatitis [4]. Direct killing of target cells is mediated by CTLs, and occurs since CTLs cannot distinguish between viruses that are or are not cytopathic.

1.3. Fatty Acids

1.3.1. Nomenclature and Dietary Sources

Fatty acids are organic compounds that contain a hydrocarbon chain of varying length, and a terminal carboxyl group [18]. They have chain lengths varying from 2 to over 30 carbon atoms, the commonest ranging between 12 and 22 [19]. They may be saturated (contain no double bonds), monounsaturated (contain one double bond) or polyunsaturated (contain more than one double bond). Chain length, the number and position of the double bond(s), and whether the double bond is in the *cis* or *trans* configuration confer different physical and chemical properties on the fatty acid.

Fatty acids are classified by three different nomenclatures; systematic naming, common naming, and shorthand notation. Systematic notation is based on the number of carbons and doubled bonds present in the acyl chain. Difficulties arise in naming unsaturated fatty acids, as the double bond may be in several different positions in the acyl chain, and even in different orientations (*cis* or *trans* configuration), and these must therefore be clearly identified [18]. Alternatively, fatty acids can be given a common name, or be described by shorthand notation, which describes the number of carbons present in the acyl chain (*a*) and the number of double bonds present (*b*) in the form *a* : *b*. In the case of unsaturated fatty acids, the position of the first double bond is also identified, in the form *ω-x*, where *x* denotes the carbon on which it appears, counted from the methyl group. *ω-x* can also be written as *n-x* (Figure 1-11). Thus, oleic acid, which has 18 carbons and one double bond on the ninth carbon, is denoted by 18:1 *n-9*. Table 1-3 lists the names and sources of common fatty acids.

Table 1-3: Nomenclature and sources of fatty acids. Taken from Calder and Burdge [18].

Systematic name	Common name	Shorthand notation	Sources
Ethanoic	Acetic	2:0	Fermentation of fibre in colon
Propanoic	Propionic	3:0	Fermentation of fibre in colon
Butanoic	Butyric	4:0	Fermentation of fibre in colon; Milk
Hexanoic	Caproic	6:0	Milk
Octanoic	Caprylic	8:0	Milk
Decanoic	Capric	10:0	Milk; Coconut oil
Dodecanoic	Lauric	12:0	Milk; Coconut oil
Tetradecanoic	Myristic	14:0	Milk
Hexadecanoic	Palmitic	16:0	Milk; Eggs; Animal fats; Meat; Cocoa butter; Palm oil (other vegetable oils contain lesser amounts); Fish oils
Octadecanoic	Stearic	18:0	Milk; Eggs; Animal fats; Meat; Cocoa butter
cis 9-Hexadecenoic	Palmitoleic	16:1 *n*-7	Desaturation of Palmitic acid; Fatty fish; Fish oils
cis 9-Octadecenoic	Oleic	18:1 *n*-9	Desaturation of stearic acid; Milk; Eggs; Animal fats; Meat; Cocoa butter; Most vegetable oils, especially olive oil
trans 9-Octadecenoic	Elaidic		Animal (ruminant) fats
cis 9-Octadecenoic	Vaccenic		
trans 11-Octadecenoic	*trans* Vaccenic		Animal (ruminant) fats
cis 9, *cis* 12-Octadecadienoic	Linoleic	18:2 *n-6*	Cannot be synthesised in mammals; Some milks; Eggs; Animal fats; Meat; Most vegetable oils, especially corn, sunflower, safflower and soybean oils; Green leaves
All-*cis* 9,12,15-Octadecatrienoic	α-Linolenic	18:3 *n-3*	Cannot be synthesised in mammals; Green leaves; Some vegetable oils, especially rapeseed, soybean and linseed oils
All-*cis* 6,9,12-Octadecatrienoic	γ-Linolenic	18:3 *n-6*	Synthesised from linoleic acid; Borage and evening primrose oils
All-*cis* 11,14,17-Eicosatrienoic	Mead	20:3 *n*-9	Synthesised from oleic acid; Indicator of essential fatty acid deficiency
All-*cis* 8,11,14-Eicosatrienoic	Dihomo-γ-linolenic	20:3 *n-6*	Synthesised from γ-linolenic acid
All-*cis* 5,8,11,14-Eicosatetraenoic	Arachidonic	20:4 *n-6*	Synthesised from linoleic acid via γ-linolenic and dihomo-γ-linolenic acids; Meat
All-*cis*-5,8,11,14,17-Eicosapentaenoic	Eicosapentaenoic	20:5 *n-3*	Synthesised from α-linolenic acid; Fatty fish; Fish oils
All-*cis*-7,10,13,16,19-Docosapentaenoic	Docosapentaenoic	22:5 *n-3*	Synthesised from α-linolenic acid via eicosapentaenoic acid; Fatty fish; Fish oils
All-*cis*-4,7,10,13,16,19-Docosahexaenoic	Docosahexaenoic	22:6 *n-3*	Synthesised from α-linolenic acid via eicosapentaenoic acid; Fatty fish; Fish oils

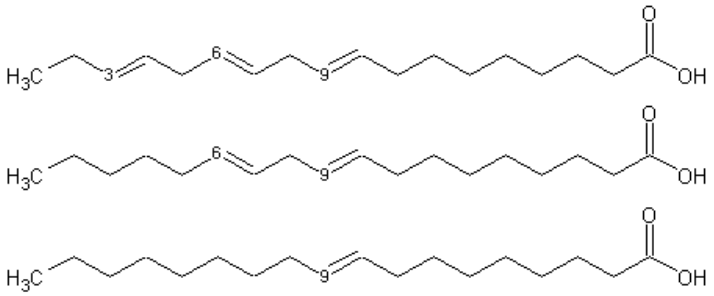

Figure 1-11: Examples of *n-3* (top), *n-6* (middle) and *n-9* (bottom) unsaturated fatty acids. Unsaturated fatty acids are classified according to the carbon on which the double bond nearest to the methyl group is positioned. Thus, *n-3* PUFAs have their double bond nearest to the methyl group on carbon three, while *n-6* PUFAs have their closest double bond on carbon six.

Whilst most double bonds in naturally-occurring fatty acids tend to be in the *cis* configuration, *trans* double bonds do occur (e.g. in intermediates of fatty acid biosynthesis, in ruminant fats, cows' milk, plant lipids and some seed oils) [18].

1.3.2. States of Fatty Acids in the Body

Fatty acids are absorbed from the diet, and circulate through the bloodstream in four main states; as triacylglycerols, phospholipids, cholesteryl esters and non-esterified fatty acids.

1.3.2.1. Triacylglycerols (TAGs)

Triacylglycerols (TAGs, triglycerides) are composed of three fatty acids attached by ester bonds to a molecule of glycerol (Figure 1-12). Most of the fat eaten, and stored by humans is in this form [20-22].

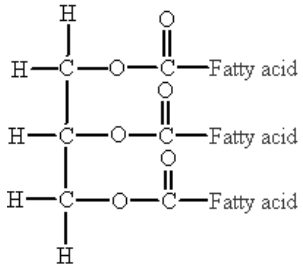

Figure 1-12: The general structure of a triacylglycerol. Triacylglycerols consist of three fatty acids attached to a glycerol molecule.

The *sn* (stereospecific numbering) system is a useful method of identifying specific carbon molecules to which fatty acids are attached. With the hydroxyl group on the middle carbon of the glycerol group drawn to the left, the carbon above it becomes *sn*-1, the middle carbon *sn*-2 and the lower carbon *sn-3* (Figure 1-13).

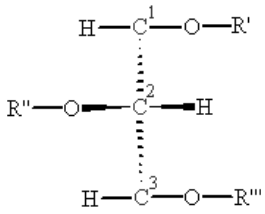

Figure 1-13: Stereospecific numbering (*sn*) system for substituted glycerides. With the hydroxyl group of the middle carbon drawn to the left, the carbon above becomes carbon one, and the R-group (in this case R') is said to be at position *sn*-1. The middle carbon is carbon two (and R'' is at position *sn*-2), and the bottom carbon is carbon three (R''' is therefore at position *sn*-3).

1.3.2.2. Phospholipids (PLs)

Phospholipids (PLs) consist of two fatty acids attached usually by ester bonds to the *sn*-1 and *sn*-2 positions of a glycerol molecule with a head group that includes a phosphate molecule attached at the s*n-3* position (Figure 1-14). The head group of PLs can vary. There are five main PL classes, as shown in Table 1-4.

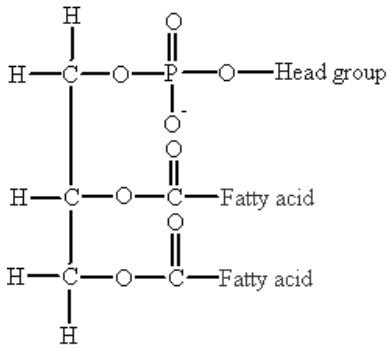

Figure 1-14: The general structure of phospholipids. Phospholipids consist of two fatty acids attached to a glycerol backbone, with a phosphate group and a variable head group (see text).

Table 1-4: Major phospholipid classes. Taken from Burdge *et al.* [23].

Name	Head group	Location
Phosphatidylcholine	Choline	Outer leaflet of cell membranes, monolayer enclosing plasma lipoproteins.
Phosphatidylethanolamine	Ethanolamine	Inner leaflet of cell membranes, small amount in plasma.
Phosphatidylserine	Serine	Inner leaflet of cell membranes
Phosphatidylglycerol	Glycerol	Inner leaflet of cell membranes, pulmonary surfactant
Phosphatidylinositol	Inositol	Anchor for proteins in the cell membranes. Polyphosphorylated forms important in cell signalling

PLs are important components of membranes, and form effective barriers. The fatty acid composition of phospholipids can alter the physical and chemical properties of the compound, and therefore affect membrane fluidity.

1.3.2.3. Cholesteryl Esters (CEs)

Cholesterol and cholesteryl esters (CEs) are also important components of membranes, as well as being precursors of vitamin D, steroid hormones and bile salts. The general structure of a CE is shown in Figure 1-15.

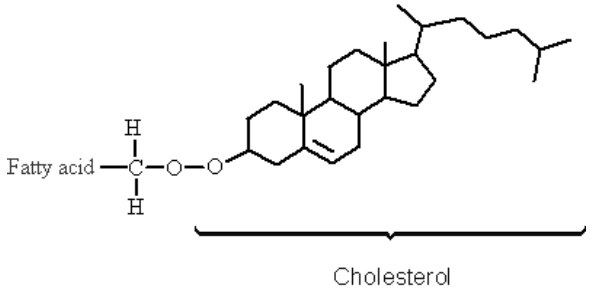

Figure 1-15: The general structure of a cholesteryl ester.

1.3.2.4. Non-Esterified Fatty Acids

Non-esterified fatty acids (NEFAs, sometimes called free fatty acids) are attached non-covalently to albumin in the blood; this allows the otherwise insoluble fatty acids to become soluble, and be transported throughout the body. Each molecule of albumin has binding sites for around three NEFAs [24]. NEFAs have a very short half-life in the blood, and are predominately used as an energy source [18]. Plasma NEFA concentration varies through the day, with levels inversely reflecting those of plasma glucose and insulin [24].

1.3.3. Digestion and Absorption of Fatty Acids

TAGs are digested in the stomach and small intestine by gastric lipase (formerly called lingual lipase) and pancreatic lipase, respectively, to form NEFAs and 2-

monoacylglycerols. There are also lipases present in breast milk that digest milk fats in neonates [21]. Pancreatic lipase is quantitatively the most important enzyme in TAG hydrolysis after weaning, whilst in infants, gastric lipase is more important [24]. A co-lipase is secreted from the pancreas, and is required to ensure pancreatic lipase binds to the lipid / aqueous interface so that it can hydrolyse TAGs [21]. Cholesterol is absorbed only as free cholesterol, and CEs must therefore be hydrolysed by cholesterol esterase before absorption. PLs are hydrolysed by phospholipases in the small intestine to form a fatty acid and lysophosphatidylcholine.

Lipid digestion products are solubilised with bile salt micelles, producing a form more readily absorbed. Short and medium-chain NEFAs in the intestinal epithelial cells move directly into the blood, where they circulate bound to albumin [24]. The remaining products of lipid digestion are re-synthesized into their former compounds (mainly TAGs), and are complexed with apolipoproteins to form chylomicrons. Chylomicrons are absorbed into the lacteal glands, from where they can circulate in the lymphatic system or pass into the blood via the thoracic duct. Chylomicrons in the blood bind to lipoprotein lipase (LPL) on the endothelial cell surface of capillaries of tissues such as adipose tissue and muscle [18, 24]. LPL hydrolyses chylomicrons to release fatty acids which can then be re-esterified inside the tissue for storage or used for oxidation [24]. LPL activity is regulated by diet and hormones such as insulin, such that activity is high in the fed state and low in the fasted state [24]. This allows dietary fatty acids to be channelled to adipose tissue rather than peripheral tissues [18]. Chylomicrons are the main form in which dietary fatty acids are transported [24].

Lipoproteins are large complexes of lipid and protein, which differ in composition and metabolic functions [18]. In addition to chylomicrons, there are three other main classes of lipoproteins. These are very-low-density-lipoprotein (VLDL), low-density-lipoprotein (LDL), and high-density-lipoprotein (HDL). As density of these lipoproteins increases, particle size decreases, as do the ratio of lipid to protein and ratio of TAGs to PLs and cholesterol [18]. Lipoprotein characteristics are shown in Table 1-5.

Table 1-5: Characteristics of the major lipoprotein classes. There are other lipoprotein fractions and sub fractions not shown h lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TAG, triacylglycerol; CE, cholesterol ester; PL, phosp

Fraction	Density range (g/ml)	Diameter (nm)	Major lipids	Major apolipoproteins[1]	Composition	
					Protein	TAG
Chylomicrons	<0.950	80 – 1000	Dietary TAG	B48, AI, AII, C, E	1	90
VLDL	0.950 – 1.006	30 – 80	Endogenous TAG	B100, C, E	10	65
LDL	1.019 – 1.063	20 – 25	Cholesterol and CE	B100	20	10
HDL	1.063 – 1.210	9 – 15	CE and PL	AI, AII, C, E	50	2

[1] Apolipoprotein C refers to the presence of apolipoproteins CI, CII, and CIII, which are usually found together.

[2] Proportions shown are approximate and vary within each major class.

VLDL is synthesized in the liver. As with chylomicrons, its main role is delivery of TAGs to tissues. LDL is formed by repeated hydrolysis of VLDL. During this process, an intermediate product is formed, known as intermediate-density lipoprotein (IDL); IDL has a short half life in the body and is usually found at low concentrations [24]. LDL is the means by which cholesterol is carried to peripheral tissues, and is the lipoprotein responsible for the deposition of cholesterol in the arterial wall in atherosclerosis. HDL is responsible for the reverse process; it removes cholesterol from tissues and transports it to the liver for catabolism.

1.3.4. Biosynthesis of Fatty Acids

Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and some polyunsaturated fatty acids (PUFAs) can be synthesized in the body.

1.3.4.1. SFA Biosynthesis

All mammals can synthesize SFAs de novo in a cyclical series of reactions in which two carbon units (derived from acetyl CoA) are added to a growing carbon chain. The main sites for this process are the liver, adipose tissue, and lactating mammary glands [18, 21, 24].

Acetyl CoA is formed in the mitochondria, and transported to the cell cytosol, where it is carboxylated to malonyl CoA by the enzyme acetyl-CoA carboxylase. Malonyl CoA and acetyl CoA then undergo a condensation reaction, followed by reduction and dehydration reactions and then a further reduction reaction. These steps are catalysed by a group of enzymes that collectively form the multi-enzyme complex fatty acid synthase [19]. This is shown in Figure 1-16. The end-product of these reactions is usually palmitic acid (16:0), with some stearic acid (18:0) formed by elongation reactions [18, 19]. However, enzymes are present in certain tissues that act to release medium chain saturated fatty acids. For example, caprylic acid (8:0) and capric acid (10:0) are produced in mammary glands of some species, and are characteristic fatty acids of their milk [18, 21].

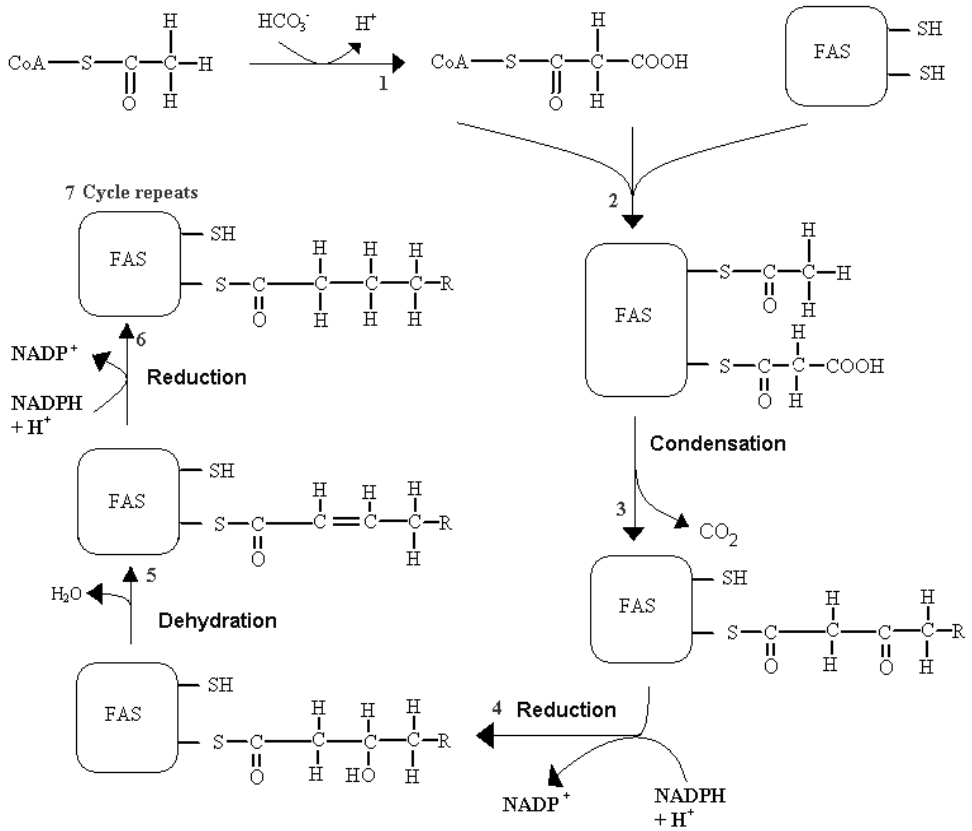

Figure 1-16: Fatty acid biosynthesis. Acetyl CoA is carboxylated to form malonyl CoA by acetyl CoA carboxylase (1). Acetyl CoA and malonyl CoA attach to the enzyme fatty acid synthase (FAS) (2) where they are condensed to form acetoacetyl ACP (3). FAS then catalyses a series of reactions so that the fatty acid is reduced (4), dehydrated (5) and further reduced (6) to form a four carbon saturated fatty acid. The cycle then repeats (7) to allow the further addition of two carbons from malonyl CoA to the growing carbon chain. Thus, on the first cycle the R group is H, on the second cycle the R group is CH_2CH_3, the third cycle the R group is $CH_2CH_2CH_2CH_3$ and so on. Adapted from Murray *et al.* [25]

1.3.4.2. MUFA Biosynthesis

SFAs can be converted to MUFAs through the introduction of a double bond. This process is known as desaturation and is carried out by the enzyme Δ9-desaturase (also known as stearoyl CoA desaturase or SCD), as the double bond is usually inserted between carbons 9 and 10 [19]. It allows the production of MUFAs of the *n-9* and *n-7* family. Examples include the conversion of stearic acid (18:0) to oleic acid (18:1 *n-9*), and of palmitic acid (16:0) to palmitoleic acid (16:1 *n-7*) [18]. This process occurs in the endoplasmic reticulum and has been identified in a wide range of organisms (bacteria, yeasts, algae, higher plants, protozoa and animals); rates of desaturation are usually quite rapid [18, 19].

1.3.4.3. PUFA Biosynthesis

All eukaryotes and some bacteria are able to produce PUFAs. Mammals, unlike plants, do not have the enzymes required to insert a double bond between carbon 9 and the methyl group in the acyl chain, and hence are unable to synthesize the simplest *n-3* or *n-6* PUFAs *de novo*. However, other *n-3* and *n-6* PUFAs can be synthesized by metabolism of α-linolenic acid (ALA) or linoleic acid (LA) respectively (Figure 1-17). These two fatty acids are therefore known as essential fatty acids, as their presence is required in the mammalian diet.

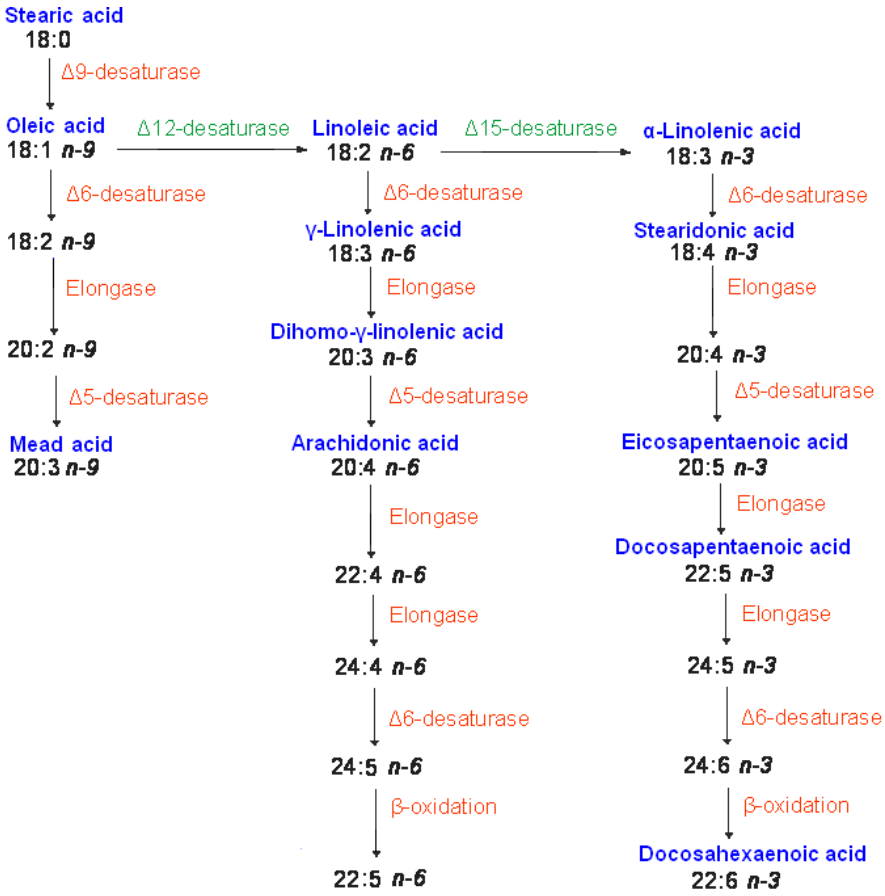

Figure 1-17: Biosynthetic pathway of polyunsaturated fatty acids. Stearic acid and oleic acid are absorbed from the diet or created by *de novo* synthesis. Enzymes are shown in red and green - those in green only occur in plants, whilst red enzymes are present in both plants and mammals. Adapted from Calder and Burdge [18].

The elongation and desaturation reactions shown in Figure 1-17 occur in the endoplasmic reticulum of the cell; β-oxidation (retroconversion) occurs in peroxisomes [21]. In mammals, the cells of the liver are able to carry out the complete pathway shown in Figure 1-17, and this is the main site of elongation and desaturation [18]. Therefore the liver converts ALA to docosahexaenoic acid (DHA), and LA to arachidonic acid (AA); these products can then be circulated to other tissues [21].

All three unsaturated fatty acid families require the Δ6-desaturase enzyme to catalyse the first step in the pathway in Figure 1-17, and this is the rate-limiting step in PUFA biosynthesis [19]. The preferred substrate of the enzyme is ALA, followed by LA, and finally oleic acid [19]. However, the level of LA in the human diet is usually high enough to overcome the problem of substrate preference, and allow significant amounts of AA to be produced [19]. Indeed, the pathway of *n-6* PUFA metabolism is quantitatively the most important. In the absence of the essential fatty acids ALA and LA, oleic acid has reduced competition for metabolism by Δ6-desaturase, and mead acid accumulates in tissues. This end-product is usually present in trace amounts, and as such, its presence at high levels can be used as an indicator of essential fatty acid deficiency [18, 19, 21].

1.3.5. Roles of Fatty Acids in the Body

Fatty acids are metabolic fuels (storage, transport and release of energy), components of cell membranes, and regulators of gene expression. The longer chain fatty acids AA, dihomo-γ-linolenic acid (DGLA), eicosapentaenoic acid (EPA) and DHA are also precursors for lipid mediator synthesis. In addition, complex lipids (esters of long chain fatty acids) provide mechanical protection as well as thermal and electrical insulation, and free fatty acids form micelles and function as soaps and detergents, due to their amphipathic nature [20].

1.3.5.1. Fatty Acids in Thermogenesis

There are two types of adipose tissue in the body, known as white and brown adipose tissue. White adipose tissue (WAT) comprises the majority of adipose tissue in adult humans [24]. In WAT, fat is stored as a single droplet, which usually almost fills the cell. The cytoplasm, mitochondria and nucleus are confined to a thin layer around the outside. Brown adipose tissue (BAT), however, has a different physiological structure from WAT. Fat is stored as multiple lipid droplets in BAT cells, and mitochondria are considerably more numerous compared to WAT, giving rise to the characteristic brown colour of the tissue.

Both BAT and WAT are storage sites for lipids. However, BAT has an additional role - producing heat. This is achieved by uncoupling proton flow from ATP production. Mitochondria pump H^+ ions from out of the mitochondrial matrix, and into the site between the two membranes, thereby creating a proton gradient. Protons return to the matrix through the enzyme ATP synthase, which converts ADP and inorganic phosphate to

ATP. In BAT mitochondria, there is a protein channel known as uncoupling protein 1 (UCP1, formerly known as thermogenin), which allows the H^+ ions to return to the matrix, and produces heat. Thermogenesis by BAT is not a constitutive process; it is induced by the sympathetic nervous system [24]. Nor is it universal; it is found in hibernating animals and infants, both of which have a real necessity for heat production [24]. However, in contrast to human infants, adult humans do not appear to have significant amounts of BAT, and for them heat loss is a greater issue than heat generation [24].

1.3.5.2. Fatty Acids as Metabolic Fuels

Fatty acids are stored in adipose tissue as TAGs. During conditions of low-energy availability, or high-energy demand (growth, reproduction or sustained mechanical work) fatty acids are hydrolysed from TAGs in adipose tissue by triacylglycerol lipase (hormone-sensitive lipase, HSL). This releases NEFAs, which then circulate bound to albumin and are transported to other tissues [18, 19, 24]. HSL activity is inhibited by insulin, so that during conditions of high fatty acid availability (i.e. following a meal), fatty acids are stored in adipose tissue rather than being mobilised from it for metabolism [24]. NEFAs are important metabolic fuels during conditions of low carbohydrate availability, such as during starvation and endurance exercise [18, 24].

Fatty acids are oxidised in the mitochondrial matrix after conversion to their fatty acyl CoA derivatives. Short- and medium-chain fatty acid CoAs readily pass into the matrix. Long-chain fatty acyl CoAs, however, require carnitine palmitoyl transferase for their transport. Fatty acid oxidation (β-oxidation) consists of the progressive removal of acetyl CoA from the carboxyl end of the fatty acyl CoA [18] (Figure 1-18). Fatty acid oxidation is overall the reverse of fatty acid synthesis, with oxidation, hydration, oxidation and cleavage reactions occurring in oxidation, as opposed to the condensation, reduction, dehydration and reduction reactions that occur in fatty acid synthesis.

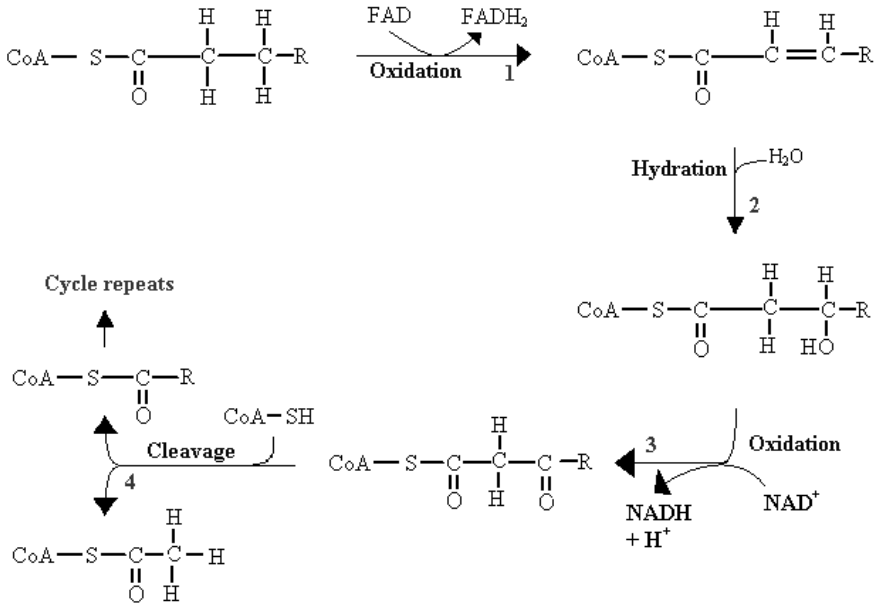

Figure 1-18: Fatty acid oxidation. This cyclical process consists of the release of acetyl CoA from the fatty acyl CoA molecule by a series of oxidation (1 and 3), hydration (2), and cleavage (4) reactions. Reaction 4 produces an acetyl CoA molecule and a fatty acyl CoA. Acetyl CoA is used in the tricarboxylic acid cycle, whilst the fatty acyl CoA undergoes another repeat of the fatty acid oxidation cycle. This continues until two acetyl CoA molecules are the sole products of reaction 4. Adapted from Murray *et al.* [25].

ATP is produced by the acetyl CoA molecules passing through the tricarboxylic acid cycle. Fatty acids produce on average 37 kJ/g (9 kcal/g) [19]. β-oxidation also occurs in peroxisomes. Peroxisomal β-oxidation has a greater specificity than mitochondrial β-oxidation, but is particularly involved in oxidation of long-chain fatty acids [25].

1.3.5.3. Fatty Acids in Membranes

The widely held understanding of cell membrane structure is the fluid mosaic model, proposed by Singer and Nicholson [25, 26]. This suggests that the membrane consists of a bilayer of lipids, with proteins embedded into it, resulting in an asymmetric, or "mosaic" structure. In addition, the membrane is "fluid", as lipids can rapidly move across the membrane (lateral diffusion) as well as rotate. To a lesser and slower extent, lipids can also move from one side of the bilayer to the other (flip-flop).

The major lipids in membranes are glycerolipids [27]. Glycerolipids can be classified into two groups; phosphoglycerides (which contain phosphorous and are also known as phospholipids) and glycosylglycerides, which contain a sugar constituent, but no phosphorous [28]. However, some compounds can be classified in both groups as they contain phosphorous and sugar residues, e.g. sphingomyelin. Phospholipids predominate in higher animals, whilst glycosylglycerides are more widespread in plants [28]. Both phospholipids and glycosylglycerides contain fatty acids esterified at the *sn*-1 and *sn*-2

positions [28]. A second major class of lipids present in membranes are sphingolipids, which contain a sphingosine backbone rather than glycerol, and fatty acids as side chains [28]. These lipids are particularly widespread in nervous tissue [28]. Examples of sphingolipids include sphingomyelin, sphingosine and ceramide; some of these act as signalling molecules. Sterols such as cholesterol are important in membranes, and can also be found in less abundantly in mitochondria, Golgi complexes and nuclear membranes [25]. Cholesterol provides stability to membranes [28].

A further role for fatty acids in membranes, and an update to the fluid mosaic model, are lipid rafts. These are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-rich domains, and are areas of the membrane involved in cellular processes such as signal transduction [29]. Lipid rafts have a different lipid composition from the rest of the membrane (high in cholesterol and glycosphingolipids), and not all rafts have the same composition of proteins and / or lipids. They are more fluid than other areas of the membrane [30]. Lipids provide anchors for proteins, and there are four major types of covalently bound lipids for membrane proteins (Table 1-6). Of these, only myristoylation and palmitoylation involve fatty acids directly (myristic and palmitic acid, respectively). Caveolae are a subclass of lipid rafts that contain caveolin-I, a cholesterol-binding protein.

Table 1-6: Lipid anchors for proteins. Taken from Gurr *et al.* [28].

Type	Attachment	Examples
Myristoylation	Co-translational; glycine at N-terminus	cAMP protein kinase; G-proteins (Go, Gi); various viral proteins
Palmitoylation	Post-translational; cysteine in body of protein	Rhodopsin; fibronectin; Ca^{2+} -ATPase
Prenylation	Post-translational; cysteine near C-terminus. Separate enzymes for farnesyl or geranylgeranyl groups. Often carboxymethylated	*Ras* proteins; G-protein (γ subunits); lamin B
GPI	Post-translational; at C-terminus; sensitive to phospholipase C	Alkaline phosphatise; 5' – nucleotidase; acetylcholinesterase

The fatty acid composition of membranes is often characteristic for the cell and membrane type, but can be affected by numerous factors, including stage of the cell cycle, age, presence of stimuli, environment and diet [25, 31]. Changes in membrane composition can cause changes in function. The fatty acid composition of cell membranes affects their

fluidity. An increased number of double bonds in unsaturated fatty acids, and / or increase in *cis*-bonds mean that membrane lipids are less tightly packed and therefore more fluid [19, 25]. This in turn can affect cell function, as fluidity is necessary for appropriate interactions between membrane proteins or proteins and lipids to occur, for example in receptor functioning, enzyme activity, transporters and signal transduction mechanisms [18]. Other lipid factors that can affect membrane functional ability include the type of lipid head group present (as this alters surface charge and potential), chain length of fatty acyl groups (must be the correct for the size of the transmembrane protein sections) and cholesterol inclusion (affects lipid lateral mobility) [28].

1.3.5.4. Fatty Acids and Gene Regulation

In addition to those described previously, fatty acids have further roles in cell signalling. For example, phospholipases C and D hydrolyse membrane phospholipids to produce diacylglycerols (DAGs) and phosphatidic acid, both of which are second messengers [18]. Cell signalling by fatty acids can also affect gene expression indirectly, as they are involved in the regulation of transcription factors. An example of this is the transcription factor family peroxisome proliferation-activated receptors (PPARs), whose ligands are fatty acids or their derivatives. There are three main isoforms of PPARs, as shown in Table 1-7. Fatty acids have different potencies in activating PPARs. For example, *n-3* PUFAs are more effective than *n-6* PUFAs, and PUFAs are more potent than SFAs [24].

Table 1-7: The main classes of peroxisome proliferation-activated receptors (PPARs). The isoforms PPAR-γ1 and PPAR-γ2 are produced from the same gene by different promoters. CPT-1, carnitine palmitoyltransferase-1; FAAR, fatty acid activated receptor; FABP, fatty acid binding protein. Taken from Frayn [24].

Receptor	Other names	Main tissue distribution	Genes whose expression is increased by PPAR activation	Genes whose expression is suppressed by PPAR activation
PPAR-α		Liver (main site). Also kidney, heart, muscle, brown adipose tissue	Apolipoprotein AI Apolipoprotein AII Enzymes of peroxisomal fatty acid oxidation Liver FABP CPT-1 Enzymes of mitochondrial fatty acid oxidation	Apolipoprotein CIII
PPAR-δ	PPAR-β, NUC 1, FAAR	Widespread	Not known, although HDL concentrations increase with activation	Not known
PPAR-γ1		Widespread at low levels		
PPAR-γ2		Adipose tissue	Factors involved in adipocyte differentiation Adipose tissue FABP Lipoprotein lipase Fatty acid transport protein Acyl-CoA synthase GLUT4 Phosphoenolpyruvate carboxykinase	Leptin

1.3.5.5. Fatty Acids and Eicosanoid Production

The PUFAs DGLA, AA and EPA can be converted to eicosanoids, following their release from membrane phospholipids (Figure 1-19). Stimuli inducing their production include histamines, cytokines, and proteolytic and lipolytic enzymes [32].

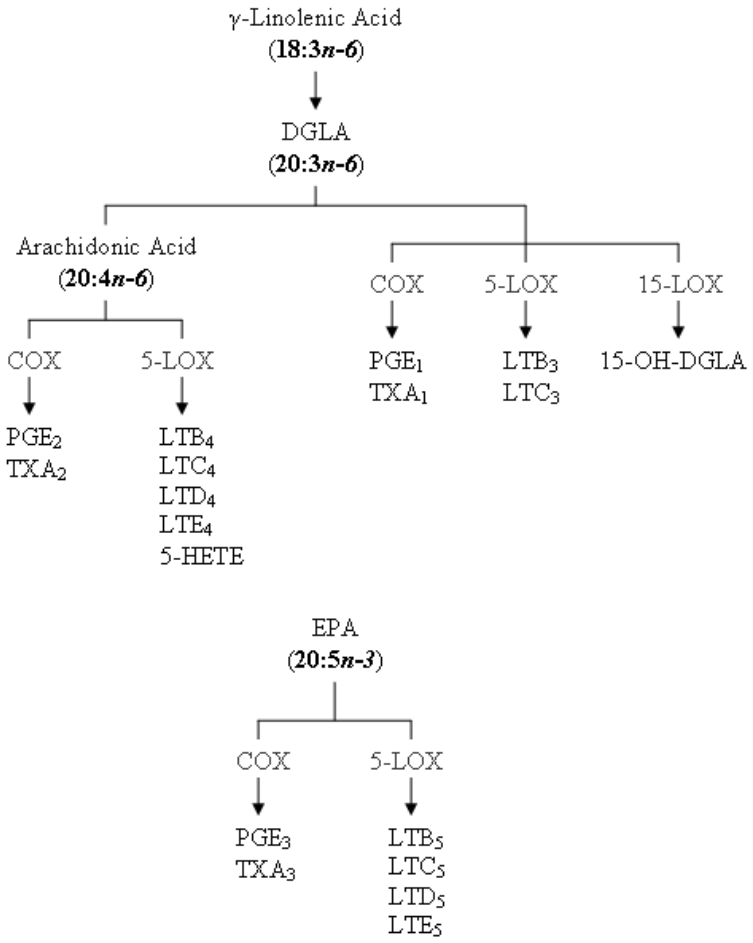

Figure 1-19: Synthesis of eicosanoids from γ-linolenic acid and arachidonic acid (top), and eicosapentaenoic acid (EPA) (bottom). COX, cyclo-oxygenase; DGLA, dihomo-γ-linolenic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotrienes; PG, prostaglandin; TX, thromboxane.

Eicosanoids are oxygenated fatty acids present in low concentrations in the body [21], and comprise prostaglandins, prostacyclins, thromboxanes, leukotrienes and lipoxins. The type of eicosanoid produced depends on which enzyme the cell expresses (i.e. COX (cyclo-oxygenase), 5-LOX, (lipoxygenase) or 15-LOX); these enzymes are present in all mammalian cells except erythrocytes [32]. Eicosanoids induce many important physiological effects (Table 1-8), such as those seen in immune responses (e.g. inflammation, pain, fever).

Table 1-8: Physiological effects of some eicosanoids. PG, prostaglandin; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; LX, lipoxin; SRS-S, Slow-reacting substance of anaphylaxis. Taken from Fernandez *et al.* [32].

Response	Eicosanoid
Vasodilation, capillary permeability	PGA, PGD, PGE_1, PGE_2, PGI_2, TXA_2
Muscle tone	PGF_2
Slow muscle contraction	LTC_4, LTD_4, LTE_4 (SRS-A)
Smooth muscle contraction	LTC_4, LTD_4
Chemoattractant for T cells	PGE_2, LTB_4
Immune suppression	LTB_4
Chemotaxis	HETEs, LTB_4, LXA
Platelet functions	TXA_2, PGI_2
Pain mediator	PGE_2, PGI_2

1.3.5.6. Prostaglandins and Derivatives

Prostaglandins all contain a five-membered ring, and are categorised according to the position of their functional groups into nine classes, denoted by names PGA to PGI. Within this, individual prostaglandins are identified using numbers, e.g. PGE_1. The main functions of prostaglandins include bronchoconstriction, inhibition of platelet-activating factor (PAF) production, cytotoxicity, and regulation of inflammation, and immunity [32]. Prostacyclin (PGI_2) causes vasodilation, and prevents platelet aggregation [33]. Thromboxanes are derivatives of prostaglandins; their effects are antagonistic to prostacyclin, causing arterial constriction and platelet aggregation [33, 34]. Thromboxanes and prostacyclin are produced by vascular smooth muscle, endothelium and platelets [32].

1.3.5.6.1. Leukotrienes

There are at least six different types of leukotrienes [35]. Leukotrienes increase permeability of small blood vessels, contract smooth muscle, and attract neutrophils to sites of infection [5, 36, 37].

1.3.5.6.2. Lipoxins

Lipoxins contain a conjugated tetraene structure and three alcohol groups; two subclasses, lipoxin A and lipoxin B, have been identified [37]. Both lipoxins A and B inhibit NK cell cytotoxicity, and lipoxin A dilates small blood vessels [37].

1.3.6. Dietary Intake of Fats

Fatty acids can be formed in the body by the elongation and desaturation reactions described in 1.3.4, but the conversion of ALA to EPA and DHA is limited in humans [38, 39], and diet is the main source of fatty acids [24]. Fats come from a variety of sources in the diet (Figure 1-20). Most fat eaten is in the form of TAGs, phospholipids, glycolipids, complex lipids and cholesterol, and fatty acids are components of all but the latter. Fatty acids therefore quantitatively constitute the majority of dietary fat [18, 24].

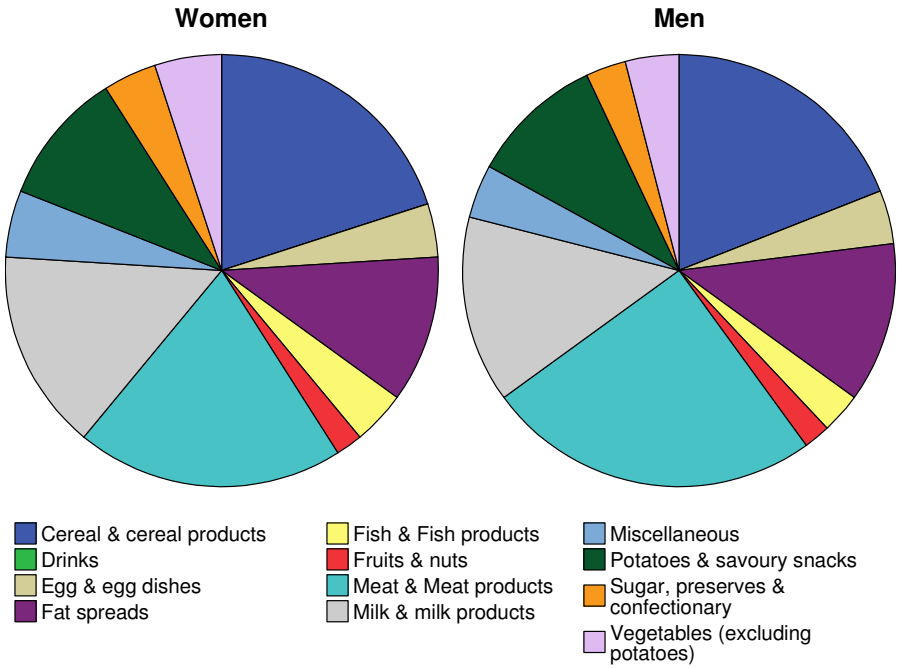

Figure 1-20: Dietary sources of total fat in the diet of British adults in the National Diet and Nutrition Survey 2000 / 2001. Constructed using data from Henderson *et al.* [40].

Dietary fat sources (e.g. milk, eggs, fish etc) have characteristic fatty acid compositions, although such compositions may be subject to alteration or variation. Cows', sheep and goats' milks contain high levels of short and medium chain fatty acids, and low levels of PUFAs. There are also small amounts of a large variety of branched and odd chain fatty acids in ruminant milks; nature and levels depend on type of feed they receive [19]. Storage fats of poultry and animals tend to be high in SFAs and MUFAs, whilst the meat contains significant levels of PUFAs [18]. Fatty acids in eggs are mostly SFAs and MUFAs found in the egg lipoproteins, whilst the yolk is rich in PUFAs in phospholipids [18, 19]. Fish can be classified into lean or oily (fatty) fish based on their fat deposition. Lean fish, such as cod, store lipids as TAGs in their liver, whilst oily fish, such as mackerel, salmon, herring and tuna, store their lipids as TAGs in their flesh. The exact fatty acid composition of fish varies according to diet and season, but fish are characteristically high in long-chain *n-3* PUFAs [19]. In some species such as herring, however, there is a

predominance of long-chain MUFAs [19]. Typical fatty acid compositions of milk, eggs, meats, animal fats and fish oils are shown in Table 1-9.

Table 1-9: Typical fatty acid composition of milk, eggs, animal fat depots, animal meats and fish oils. Data are shown as g fatty acid pe from Calder and Burdge [18].

	≤ 12:0	14:0	16:0	18:0	16:1 *n-7*	18:1 *n-9*	18:2 *n-6*	18:3 *n-3*	20:4 *n-6*	20:5 *n-*
Cows' milk	13	12	26	11	3	28	2	1	0	0
Cows' milk[a]	10	8	17	8	<1	21	35	1	0	0
Goats' milk	21	11	27	10	2	26	2	1	0	0
Sheep milk	24	12	25	9	3	20	2	1	0	0
Human milk	6	7	27	10	4	35	7	1	some	trace
Egg yolk	0	0	29	9	4	43	11	<1	some	0
Pig fat (lard)	<1	1	29	15	3	43	9	1	0	0
Poultry fat	some	1	27	7	9	45	11	0	0	0
Poultry fat[b]	some	1	22	6	5	27	35	0	0	0
Beef fat (suet)	some	3	26	5	9	45	2	1	0	0
Lamb fat	some	3	21	20	4	41	5	0	0	0
Pork	some	<1	19	12	2	19	26	1	8	0
Chicken	some	<1	23	12	6	33	18	1	6	0
Beef	some	<1	16	11	2	20	26	1	13	0
Lamb	some	<1	22	13	2	30	18	4	7	0
Herring oil[c]	some	9	15	1	7	16	1	<1	1	10
Menhaden oil[c]	some	8	22	3	11	21	2	2	2	14
Cod liver oil[c]	some	5	15	3	10	15	2	<1	1	10

[a] Cows fed supplement containing LA and protected against rumen biohydration

[b] Chickens fed diet rich in LA

[c] Also contain 4 – 40 g 20:1 *n-9* and 22:1 *n-9* per 100 g total fatty acids

The fatty acid composition of plant membrane lipids is less variable between different leaf types, with five fatty acids accounting for more than 90% of the total (palmitic, palmitoleic, oleic, linoleic and α-linolenic) [18, 19]. Green leafy vegetables are an important source of ALA in the human diet [18, 19]. In contrast to this, there is a wide variation of fatty acids in seed oils. Often, one fatty acid predominates that is characteristic to a particular species and has an unusual structure [18, 19]. Fatty acid composition of selected seed oils is shown in Table 1-10.

Table 1-10: Fatty acid composition of selected seed oils consumed by humans. Data is shown as g fatty acid per 100 g total fatty acids. R for both high and low erucic forms. Older varieties of rapeseed contain nearly 60% erucic acid, which was reported to be toxic, and so n acid (achieved through breeding) [18]. Data taken from British Nutrition Foundation [19].

Oil	8:0	10:0	12:0	14:0	16:0	18:0	20:0	22:0	16:1	18:1	18:2	18:3
Coconut	8	7	48	16	9	2	1	0	trace	7	2	0
Corn	0	0	0	1	14	2	trace	trace	trace	30	50	2
Olive	0	0	0	trace	12	2	trace	0	1	72	11	1
Palm	0	0	trace	1	42	4	trace	0	trace	43	8	trace
Palm kernel	4	4	45	18	9	3	0	0	0	15	2	0
Peanut	0	0	trace	1	11	3	1	3	trace	49	29	1
Rapeseed (Low erucic)	0	0	0	trace	4	1	1	trace	2	54	23	10
Rapeseed (High erucic)	0	0	0	trace	4	1	1	trace	trace	24	16	11
Soybean	0	0	trace	trace	10	4	trace	trace	trace	25	52	7
Sunflower	0	0	trace	trace	6	6	trace	trace	trace	33	52	trace

[a] 20:1

[b] 22:1

There are large differences in fatty acid intake between countries, ranging from under 20 g/day in some developing countries, to more than 150 g/day in certain developed countries [18]. There have also been large changes in fat consumption over time, a pattern which looks set to continue, particularly with the increase in availability of low fat foods. Figure 1-21 shows the changes in consumption of saturated and polyunsaturated fat, and vegetable oil among adults in the UK from 1966 onwards. Much of this change reflects improved consumption habits, for example changing from butter to margarine and from animal fats to vegetable oils [18, 19].

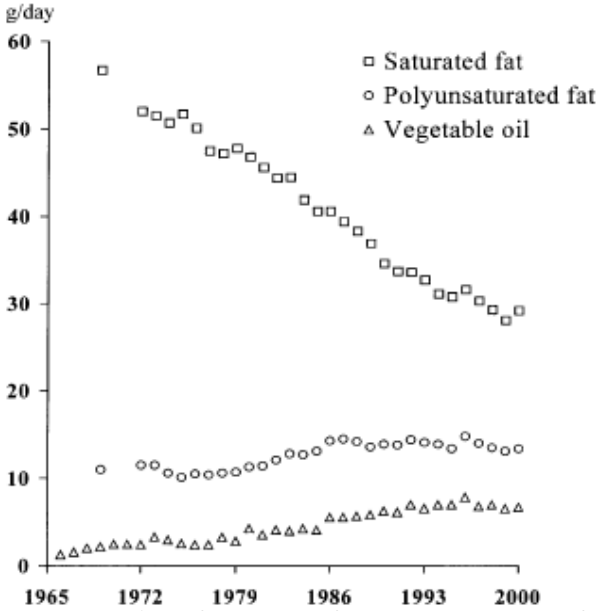

Figure 1-21: Trends in consumption of saturated fat, polyunsaturated fat and vegetable oil in the UK since 1966. Taken from Devereux and Seaton [41].

The Committee of Medical Aspects of Food [42] has made recommendations for fat intake in the UK, expressed as average population values. These are shown in Table 1-11, along with the average intakes in British adults in 2000/2001. On average, 13.5 g of LA and 1.7 g of ALA is consumed by adult men in the UK daily; for women these figures are 9.3 g and 1.2 g respectively [18].

Table 1-11: Fat recommendations in the UK, shown as percentage of energy both including and excluding alcohol [42] and the average British adult intakes in 2000 / 2001 [40]. COMA, Committee on Medical Aspects of Food Policy; FA, fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

	COMA recommendations (% energy)		Average British adult intakes (% energy)	
	Including alcohol	**Excluding alcohol**	**Men**	Women
Total fat	< 33	< 35	35.8	34.9
SFAs	< 10	< 11	13.4	13.2
MUFAs	12	13	12.1	11.5
cis PUFAs	6 - 10	6.5 - 11	6.4	6.3
trans FAs	< 2	< 2	1.2	1.2

1.4. Fatty Acids and the Immune System

In the years 1950 to 1974 in Upernavik District in Greenland there was a low incidence of asthma and related diseases [43], accompanied by a high intake of *n-3* PUFAs found in high amounts in sea mammals and fish [44]. Other epidemiological studies have shown a similarly low level of allergic disease in populations with a high fish intake [45, 46]. In contrast, in countries, such as the UK, Australia, New Zealand, Sweden, Finland, the United States and Canada, there has been an increased incidence of allergies and related diseases, accompanied by an increased consumption of *n-6* PUFAs [47]. These observations lead to the idea that dietary fat, and in particular the balance of *n-3* and *n-6* PUFAs, may affect allergy development [47, 48] as a result of the different effects of these classes of fatty acids on immune cell functions according to their properties described in section 1.2.5. In fact, different fatty acids have been shown to affect immune cell functions and immune responses in many model systems; it is not possible to review the entirety of this literature here but extensive reviews may be found elsewhere [49-51]. In the sections below, the effects of fatty acids on immune functions relevant to the studies conducted in this thesis will be described. The earlier work has used a) cell culture experiments where isolated cells have been exposed to individual fatty acids, b) studies in laboratory rodents where the fat composition of the diet has been manipulated, c) studies in humans where specific fats or fatty acids have been provided most often in the form of capsules consumed on a daily basis. Each of these approaches has advantages and disadvantages. One difficulty of interpreting the findings from many of these studies, irrespective of the approach used, is the nature of the control. For example in an animal study investigating the effect of an increase in dietary *n-3* PUFAs, it is not entirely clear whether the control should be a diet rich in saturated fat or in *n-6* PUFAs or representing some sort of balanced fatty acid composition and different effects of the *n-3* PUFA-rich diet may be seen in comparison to each of these other diets.

1.4.1. T-Cells

1.4.1.1. Effect of SFAs

Diets high in SFAs have been shown not to affect lymphocyte proliferation compared to low-fat diets [52], to reduce proliferation but less so than high PUFA diets [53-60] and even to increase proliferation compared to low fat and high PUFA diets [61, 62]. Moussa *et al.* [57] demonstrated that a high fat (200 g lipid / kg) coconut oil diet reduced proliferation of splenic lymphocytes compared to a low fat (50 g / kg) coconut oil diet. One

study investigated the effect of individual SFAs on lymphocyte proliferation directly: Jeffery *et al.* [63] showed that rat lymphocyte proliferation is enhanced most by a diet rich in palmitic acid in the *sn*-2 position of dietary TAGs. In cell culture, addition of stearic acid has been shown to inhibit T-cell proliferation, whilst palmitic acid and myristic acid did not significantly affect it [64]. Studies have found no effect of SFAs on levels of T-cells in the rat compared to fish oil (FO) [59, 65, 66] or LA [59, 65, 66].

1.4.1.2. Effect of *n-3* PUFAs

ALA-rich diets have been shown to have no effect on T-cell abundance in humans in comparison to low ALA [67], DHA [68] or AA [68]. Soybean oil diets have been shown to reduce lymphocyte proliferation in rats compared to low fat feeding [57, 58, 60], or coconut oil diets [57], and in mice compared to low soybean oil [69]. However, soybean oil diet experiments are complicated by the fact that they are rich in LA, as well as containing ALA, and it is therefore unclear whether effects seen are due to *n-3* or *n-6* PUFAs. Jeffery *et al.* [70] found a relationship between LA and splenic lymphocyte proliferation, and an inverse relationship between level of ALA and splenic lymphocyte proliferation in rats although neither relationship was linear. Feeding ALA-rich diets suppresses spleen T-cell proliferation compared to feeding diets low in ALA [67, 71], diets rich in HCO [62], sunflower oil [71], canola oil, corn oil and lard [72]. However, some researchers have found no significant effect of ALA-rich diets in humans on lymphocyte proliferation compared to diets low in ALA [68, 73]. In cell culture, addition of ALA has been shown to inhibit T-cell proliferation [64].

EPA has been shown to have no effect on level of T-cells in humans [74] or rats [75, 76] compared to SFAs[74], ALA [75, 76], DHA [75], AA [75] or GLA [74, 75]. DHA has been shown to have no effect on absolute number or proportion of lymphocytes that are T-cells in humans [68, 77] or rats [75, 76] compared to ALA [68, 75, 76], EPA [75], FO [68], LA [77], AA [68, 75], or GLA [68, 75]. Increased consumption of fish as part of a low fat diet has been found to have no effect on level of T-cells in humans [78]. FO has been shown to have no effect of absolute number or proportion of lymphocytes that are T-cells in humans [68, 79] or rats [59, 65, 66] compared to SFAs [65], ALA [68], DHA [68], LA [59, 65, 66, 79], GLA [59, 65, 66, 68, 79] or OA [59, 65, 66, 79].

Feeding FO to rabbits [80], chickens [72], rats [59, 66, 81, 82], or mice [61] suppresses T-cell proliferation compared to feeding low fat [59, 66, 78, 81], HCO [61, 66, 82], lard [72], linseed [80], safflower [59, 66, 82], or corn oils [72]. Fish or FO supplementation in humans has also been reported in some studies to suppress lymphocyte proliferation compared to baseline

values [68, 78, 83-85] or high SFA+MUFA+*n-6* PUFA intake [86]. However, other studies have shown an increase in T-cell proliferation as a result of FO provision in mice compared to corn oil [87] or baseline values [88]. EPA has been shown to reduce T-cell proliferation compared to LA supplementation [89]. Feeding DHA [68, 77], EPA [74] or both EPA and DHA [73, 79] to humans has been shown to have no effect on lymphocyte proliferation compared to SFAs [74], ALA [73], LA [77, 79] or baseline values or no additional *n-3* PUFAs [68, 73]. However, EPA and DHA have both been shown to reduce lymphocyte proliferation in rats [75, 90] and mice [91] compared to ALA [90], soybean oil [75] or LA [91]. Again, position of the fatty acid in the dietary TAG may influence its effects or lack thereof on the immune system; Kew *et al.* [76] showed that moderate supplementation of EPA in position *sn*-1(3) of TAG increased lymphocyte proliferation in rats compared to ALA. In addition, dietary supplementation with salmon (to a predicted level of 2.3 g EPA and 3.6 g DHA) has been shown to have no effect on T-cell proliferation in humans, compared to a low fish diet [92]. In cell culture, additions of both EPA and DHA have been demonstrated to inhibit human T-cell proliferation [64].

1.4.1.3. Effect of *n-6* PUFAs

Studies have shown that LA-rich diets can reduce lymphocyte proliferation in mice [53, 55, 61, 93, 94], guinea pigs [54], and rats [52, 59, 62, 66, 95] compared to low fat diets [55, 59, 66, 95] or SFA-rich diets [52-55, 61, 62, 66, 93, 94]. However, other studies report no effect of LA on lymphocyte proliferation in experimental animals [96-99]and humans [79] compared to SFAs [96, 99], ALA [97], or FO [79, 97-99]. In cell culture, addition of LA has been shown to inhibit human lymphocyte proliferation [64]. LA-rich diets have been shown to have no effect on levels of T-cells in humans [79] or rats [59, 65, 66] compared to FO [59, 65, 66, 79], or SFAs [59, 65, 66].

AA has been shown to have no effect on level of T-cells in rats compared to GLA, EPA or DHA [75] or in humans compared to a low AA diet [68, 100]. AA has been shown to have no effect on lymphocyte proliferation compared to low AA or baseline values [68, 101], in mice, compared to LA [91], or when added to cultures of human T-cells [64].

1.4.2. T-Helper Cells

1.4.2.1. Effect of SFAs

SFAs have been shown not to affect T_H cell numbers in the rat compared to a low fat diet [63], FO [59, 65, 66] or LA [59, 65, 66].

1.4.2.2. Effect of *n-3* PUFAs

ALA-rich diets or supplements have been shown to have no effect on levels of circulating T_H cells in humans [67, 68] or rats [71] compared to low ALA [67], DHA [68], FO [68], LA [71] or AA [68].

EPA has been shown to have no effect on level of T_H cells in humans [74], rats [76] or mice [91] compared to SFAs [74], ALA [76] or LA [91]. DHA supplements have been found to have no effect of levels of T_H cells in humans [68, 77] rats [76] or mice [91] compared to ALA [68, 76], FO [68], LA [77, 91] or AA [68]. Reports have suggested that FO has does not affect level of T_H cells in rats [59, 65, 66, 81] or humans [68, 79] compared to SFAs [59, 65, 66], ALA [68], LA [59, 65, 66, 79, 81] or AA [68]. Increased consumption of fish in a low fat diet has been shown to have no effect on level of T_H cells in humans [78].

1.4.2.3. Effect of *n-6* PUFAs

LA-rich diets may not affect T_H cells in humans [79] or rats [59, 65, 66, 71, 81] compared to SFAs [59, 65, 66], ALA [71] or FO [59, 65, 66, 79, 81].

1.4.3. Cytotoxic T-Cells

1.4.3.1. Effect of SFAs

SFAs do not appear to affect level of CTLs in the rat [59, 63, 65, 66] compared to a low fat diet [63], FO [59, 65, 66] or LA [59, 65, 66].

1.4.3.2. Effect of *n-3* PUFAs

ALA-rich diets or supplements have been shown to have no effect on level of CTLs in humans [67, 68] compared to low ALA [67], DHA [68], FO [68] or AA [68].

DHA supplementation has been shown to have no effect on levels of CTLs in humans [68, 77] or rats [76] compared to ALA [68, 76], LA [77] or AA [68]. However, Jolly *et al.* [91] found DHA supplementation reduced $CD8^+$ expression in mice, compared to LA supplementation. EPA has been shown to have no effect on level of CTLs in humans [74], rats [76] or mice [91] compared to SFAs [74], ALA [76] or LA [91]. FO supplementation has been shown to have no effect on level of CTLs in humans [68, 79] or rats [59, 65, 66, 81] compared to ALA [68], LA [59, 65, 66, 79, 81] AA [68] or SFAs [59, 65, 66]. However, Meydani *et al.* [78] found an increase in CTL expression in humans on a low fat, high fish diet, compared to baseline values.

1.4.3.3. Effect of *n-6* PUFAs

LA-rich diets or supplements have been shown to have no effect on level of CTLs in humans [79] or rats [59, 65, 66, 71, 81] compared to SFAs [59, 65, 66], FO [59, 65, 66, 79, 81] or ALA [71].

Reports suggest that AA has no effect on level of CTLs in humans [68, 100] or mice [91] compared to a low AA diet [91, 100], ALA [68], DHA [68] or FO [68].

1.4.4. T-Cell Derived Cytokines

1.4.4.1. Effect of SFAs

Wallace *et al.* [61] showed IL-2 and IL-4 production in mice was higher following coconut oil feeding than fish and safflower oil (SAFO) feeding; IL-2 production was also higher than low fat feeding. Addition of stearic acid to cell cultures of human lymphocytes caused a reduction in IL-2 production, whilst myristic acid and palmitic acid had no effect [64].

1.4.4.2. Effect of *n-3* PUFAs

Berger *et al.* [97] found no effect of linseed oil diets on IL-2 production in mice in comparison to SAFO or FO. ALA-rich diets or supplements have been shown to have no effect on IL-2 [67, 73, 102], IL-10 [73] or IFN-γ [73] production in humans in comparison to FO [73, 102], LA [102] or low ALA or no additional supplementation [67, 73]. The addition of ALA to cell cultures of human lymphocytes reduced IL-2 production [64].

Kelley *et al.* [77] showed no effect of supplementation of 6 g DHA per day to humans, compared to supplementation with LA. The addition of EPA or DHA to cell cultures of human lymphocytes has been shown to reduce IL-2 production [64]. However, Jolly *et al.* [91] showed that both EPA and DHA reduced IL-2 production in mice compared to LA supplementation.

Berger *et al.* [97] and found no effect of FO diets on IL-2 production in mice compared to linseed or SAFO, and Turek *et al.* [102] found no effect of FO on IL-2 production in pigs compared to corn oil or linseed oil, whilst other studies have demonstrated a reduction of IL-2 production following FO supplementation compared to baseline values [84] or coconut oil supplementation [61]. Wallace *et al.* [61] also demonstrated a reduction in IFN-γ production, but no difference in IL-4 production as a result of FO supplementation, compared to coconut oil supplementation. However, Fernandes *et al.* [87] found FO supplementation to increase IL-2 production by splenocytes from female (NZB/NZW) F1 (BW) mice (an animal model for human SLE (systemic lupus erythematosus)) compared to those receiving corn-oil based diets. The same study found no difference in IL-4 production between the two groups. In humans, FO has been shown to have no effect on production of IL-2 [73, 79], IL-4 [88], IL-10 [73, 79] or IFN-γ [73, 79] compared to ALA [73], LA [79] or no additional supplementation or baseline values [73, 88]. Other studies have shown a decrease in IL-2 [85, 103] and IFN-γ [88, 103] production as a result of FO supplementation, compared to no additional supplementation or baseline values.

1.4.4.3. Effect of *n-6* PUFAs

Diets high in LA have been shown to have no effect on IL-2 [79, 91, 97] or IL-4 [61], IL-10 or IFN-γ [79] production in mice [61, 87, 91, 97] or humans [79] compared to SFAs [61], ALA [97] or FO [79, 87, 91, 97]. In contrast, Wallace *et al.* [61] found IL-2 production was lower following feeding of *n-6* rich diets compared to high SFA diets. Yaqoob *et al.* [93] found SAFO diets increased IL-2 production in mice compared to low fat and HCO diets. The same study found no effect on IL-4, IL-10 or IFN-γ production. In cell cultures of human lymphocytes, the addition of LA has been shown to reduce IL-2 production [64].

Thies *et al.* [68] found no effect of AA supplementation on IL-2 or IFN-γ production by older humans compared to those receiving ALA, DHA or FO. Kelley *et al.* [100] found no effect of AA supplementation on IL-2 production by younger men compared to a control receiving a low AA diet. In cell cultures of human lymphocytes, the addition of AA has been shown to reduce IL-2 production [64].

1.4.5. B-cells

1.4.5.1. Effect of SFAs

Diets high in SFAs have been shown to have no effect on B-cell proliferation in mice [56, 94, 99] and cats [104] compared to diets rich in *n-3* PUFAs [104], FO diets [99] or corn

[56, 94, 99], but cause increased proliferation compared to low fat diets [56]. SFA-rich diets have been shown to have no effect on level of B-cells in rats [59, 65, 66] compared to FO [59, 65, 66] or LA [59, 65, 66].

SFA-rich diets have been shown to cause lower IgG production [53, 55, 56] but higher IgM production [55] or no difference in IgM production [53, 56] compared to LA [53, 55] or high PUFA diets [56].

1.4.5.2. Effect of *n-3* PUFAs

Olson *et al.* [69] found no difference between a high (20 g / 100 g) or low (5 g / 100 g) of soybean oil level on B-cell proliferation in mice. However, this is complicated by the presence of *n-6* PUFAs in soybean oil. ALA has been shown to increase B-cell proliferation, and antibody titres in rabbits, in comparison to FO and LA [80]. ALA-rich diets or supplements have been shown to have no effect on level of circulating B-cells [67, 68] compared to low ALA [67], DHA [68], FO [68] or AA [68], or on serum concentrations of IgG and it subclasses, IgA or salivary IgG in humans or rats, compared to LA [71].

DHA has been shown to have no effect on IgG production in humans [77], antibody titre in response to influenza in humans [105] or number of B-cells in humans compared to ALA [68] or AA [68], or in rats [75, 76] compared to ALA [76], LA [77, 105], AA [75], or EPA [75]. EPA appears to have no effect on level of B-cells in rats [75, 76] compared to ALA [76], DHA [75], or AA [75], or humans, compared to SFAs [74]. Miles *et al.* [74] also demonstrated an increase in IgG2 plasma concentration, and reduction of IgE plasma concentration of young men as a result of 2.0 g EPA supplementation for 7 weeks, at the end of supplementation compared to baseline values. FO has been shown to increase antibody titres in chickens [72] compared to corn, canola, or linseed oil, and reduce Ig+ expression in splenocytes of female (NZB/NZW) F1 (BW) mice (a model of human SLE) compared to corn oil [87]. FO appears to have no effect on levels of circulating B-cells in humans [68, 79] or rats [59, 65, 66] compared to SFAs [59, 65, 66], ALA [68], LA [59, 65, 66, 79], or AA [68]. Increased dietary supplementation with fish has been shown to have no effect on B-cell proliferation in humans [78, 92].

1.4.5.3. Effect of *n-6* PUFAs

B-cell proliferation has been shown not to be affected by diets high in *n-6* PUFAs in mice [53, 99] and chickens [72] compared to those receiving SFA diets [53, 72, 99], linseed oil [72] or FO diets [72]. However, Morrow *et al.* [56] found an increase in B-cell proliferation in mice receiving a corn oil diet compared to those receiving a lard-based diet. Friend *et al.* [54] found reduced antibody titre in guinea pigs fed a maize-oil diet compared to those on a tallow-diet. LA rich diets or supplements have been shown to have no effect on circulating levels of B-cells in humans [79] or rats [59, 65, 66, 71] compared to SFAs [59, 65, 66], ALA [71] or FO [59, 65, 66, 79].

AA supplementation to humans has been shown to have no effect on B-cell proliferation [101], antibody titre [100], or absolute or percentage numbers of B-cells in

circulation in humans [68, 100] or rats [75] compared to low AA [100, 101], ALA [68, 75], DHA [68, 75], EPA [75], or FO [68] supplementation.

1.4.6. Delayed-Type Hypersensitivity

There are numerous *in vivo* measures of immune responses, one of which are measures of delayed-type hypersensitivity (DTH) responses. DTH responses are local cell-mediated responses produced in an individual as a result of receiving an injection into the skin of an antigen they are sensitive to. 24 – 48 h after this injection, the subsequent induration can be measured to provide an *in vivo* quantification of the cell mediated response, and thus can be used to determine the effect of fatty acids on cell mediated immunity [106].

1.4.6.1. Effect of SFAs

Reports have suggested SFAs may have no effect on DTH response in mice [56, 107] and rabbits [80] compared to ALA [80], FO [80] or LA [56, 80, 107]. However, Friend *et al.* [54] demonstrated an increased DTH response in guinea pigs fed a high SFA diet compared to those on a high PUFA diet.

1.4.6.2. Effect of *n-3* PUFAs

Studies have shown both no effect of ALA in DTH response in humans in comparison to no additional *n-3* PUFAs or FO [73] or baseline values [67], and in rabbits in comparison to LA, FO or SFAs [80].

DTH response has been reported to be unaffected by EPA [74], DHA [77] or EPA + DHA in humans [73] compared to no additional *n-3* PUFAs [73], SFAs [74], ALA [73], LA [77] or GLA [74]. Similarly, DTH response has been found to not be affected by FO in rabbits, compared to LA, ALA and SFAs [80] or increased fish consumption in adults [92]. However, other studies have reported a reduction in DTH as a result of consumption of EPA or DHA compared to LA [89], FO (compared to low fish oil) [108], or no EPA [109] or increased consumption of fish [78].

1.4.6.3. Effect of *n-6* PUFAs

Studies have reported no effect on DTH response of LA-rich diets in mice [56] or rabbits [80], compared to SFA-rich [56, 80], ALA or FO [80] diets. However, Friend *et al.* [54]

found reduced DTH response in guinea pigs fed a high LA diet compared to those on a high SFA diet. Kelley *et al.* [101] found no effect of AA supplementation on DTH response in men, compared to men not receiving additional AA.

1.5. Mechanisms for the Effect of PUFAs

PUFAs can affect the immune system through several different mechanisms. PUFAs may alter the activation of intracellular signalling pathways, activation of lipid-raft-associated proteins, regulation of gene expression / activity of transcription factors, and the production of eicosanoids.

1.5.1. Intracellular Signalling Pathways

Intracellular signalling pathways may be affected by PUFAs indirectly due to the use of phospholipids to form second messengers such as DAGs, ceramide and phosphatidic acid. DAGs activate sphingomyelin ceramide-phosphohydrolase to release ceramide, and some isoforms of protein kinase C (PKC) [24]. DAGs and their metabolites also act to alter mitogen-activated-protein-kinases (MAPKs) [110], thereby affecting pathways they control, such as proliferation and differentiation [111]. Ceramide can be converted to sphingosine, which activates certain phospholipases, and sphingosine-1-phosphate [112]. Phosphatidylserine is required for PKC activation [18]. Changes in the fatty acyl chains attached to the glycerol moieties of these compounds have the potential to affect the compounds' properties, and thus intracellular signalling. DAGs synthesized with two SFAs, or a SFA and an unsaturated fatty acid have been shown to be induce less activity in PKC than dioleoylglycerol or diarachidonylglycerol [113]. Synthesizing DAGs with AA, DHA or EPA has been shown to induce differences in activation level of PKC [110, 114] and MAPKs [115]. Activity of rat spleen PKC has been shown be affected by combinations of phosphatidylserine and DAG with different fatty acid compositions [116]. Fatty acids may also play a more direct role in control of intracellular signalling, due to their modulatory effects on PKC [117].

1.5.2. Lipid-Raft-Associated Proteins

Dietary PUFAs can affect the composition of lipid rafts, further than the increased abundance of the dietary fatty acid in membranes. Fan *et al.* [118] showed that mice receiving a FO-based diet had a decreased sphingolipid content of lipid rafts compared to those receiving a corn oil-based diet. As lipid rafts are responsible for the function and localisation of many receptors involved in T-cell signalling, it follows that changes in the composition and biophysical properties of lipid rafts due to dietary fatty acids could therefore impact on the functioning and signalling of T-cells. PUFAs may act though displacing signalling proteins from lipid rafts, thus inhibiting T-cell signal transduction.

EPA treatment to cell cultures of Jurkat T-cells has been shown to reduce expression of IL-2Rα [119] on T-cells, and to displace a signalling protein called "linker for activation of T-cells", and other signalling proteins from lipid rafts [120]. Jurkat T-cells treated with a variety of PUFAs, showed a displacement of the acylated proteins attached to the inner membrane, such as the Src family of kinases (and the linker for activation of T-cells), but retained GPI-anchored proteins attached to the outer membrane [121].

1.5.3. Gene Expression

PUFAs may affect the immune system through alteration of transcription factor activity and subsequent modification of gene expression. This occurs due to a direct action on ligand-binding transcription factors (nuclear receptors), or effects on membrane or cytoplasmic signalling factors which affect transcription factor activity. Two of the main transcription factors affected by fatty acids and involved in regulation of the immune system are PPARs and NF-κB.

PPARs are nuclear receptor ligand-activated transcription factors activated by fatty acids (see 1.3.5.4). Three main forms exist, which are PPAR-α, -β, and -γ. Fatty acids have different potencies in activating PPARs; *n-3* PUFAs are more effective than *n-6* PUFAs, and PUFAs are more potent than SFAs [24]. PPARs have numerous roles in immune regulation, such as inhibition of inflammation, cellular proliferation and differentiation and apoptosis [122]. PPAR-α and -γ are expressed in dendritic cells, and T- and B-cells (reviewed by Daynes and Jones [123]). Most of these roles stem from the ability of PPARs to regulate activity of NF-κB, STATs (signal transducers and activators of transcription), AP1 (activator protein 1) and NFAT (nuclear factor of activated T-cells) (reviewed by Daynes and Jones [123]).

NF-κB is a transcription factor that has an important role in the regulation of genes involved in both the innate and adaptive immune system. It regulates the synthesis of cytokines such as IL-1, IL-2, IL-6, TNF-α and IFN-β, cytokine receptors such as IL-2R, adhesion molecules and enzymes involved in mediator synthesis. It is activated by the phosphorylation and subsequent release of IκB (inhibitory κB), one of its subunits, which allows NF-κB to move to the nucleus, and bind to the response elements on its target genes. Phosphorylation of IκB can occur through the actions of PKC, the activities of which, as stated previously, can be affected by fatty acids.

1.5.4. Eicosanoids

One link between PUFA and the immune system is through the actions of eicosanoids (see 1.3.5.5). Immune cell membranes contain more AA than DGLA or EPA, and therefore AA is usually the principal precursor for eicosanoid synthesis [51]. As can be seen in Figure 1-19, AA is converted to PGE_2 under the influence of the COX enzymes.

PGE_2 has many roles in regulating immune function including influencing T helper cell activation and proliferation (reviewed by Tilley *et al.* [124]). A mechanism underlying this appears to be that PGE_2 prevents the hypomethylation of CpG islands in the promoter region of the IFN-γ gene [125]. Since hypomethylation of the IFN-γ promoter region is required for transcription [126], this will cause a reduction in level of production of IFN-γ. IFN-γ is known to enhance the proliferation of T_H1 cells (see 1.2.3.2.2). However, substrate availability for eicosanoid synthesis will be affected by dietary availability of precursors, and this provides a means by which to manipulate immune cell abundance and function. Thus, an excess of *n-6* PUFAs in the diet may result in polarisation of T_H cells away from the T_H1 and towards the T_H2 phenotype. Since T_H2 cells promote allergic hypersensitivity, *n-6* PUFAs might increase the potential to develop allergy [47]. *n-3* PUFAs are not converted to PGE_2 and act to decrease PGE_2 production (reviewed by Calder *et al.* [127]), may therefore allow hypomethylation of IFN-γ, and potentially enhance T_H1 polarisation and reduce the likelihood of allergy development.

1.6. Programming

In 1989, Barker *et al.* proposed a link between low birth weight and high risk of developing ischemic heart disease in adulthood [128]. This research was further developed to link exposure to certain factors *in utero* with health in adulthood. The theory behind this is that by sensing environmental cues, the foetus is able to adapt itself for the environment into which it expects it will be born, thereby potentially providing advantage in the future by increasing likelihood of survival. These changes are known as predictive adaptive responses, and it is thought that phenotypic changes are more likely to have long-lasting effects if they occur in early life [129]. It is important to note, however, that the predictive adaptive responses may prove detrimental if the environment changes in later life and adaptations are irreversible [129]. As such, this theory has been further examined in several models of adult disease, for example low birth weight (data for birthweight is relatively easy to obtain from records) has been linked to increased likelihood of Type II diabetes, the metabolic syndrome and high blood lipids in adulthood [129]. The theory was then further developed to examine the effect of pre-natal nutrition on health, and the term "programming" introduced to describe the physiological changes induced by stimuli or insults during critical windows of development on health in later life [130]. These stimuli or insults may be due to them inducing, deleting or impairing the development of a somatic structure, or causing irreversible physiological changes [131].

1.6.1. Programming of the Immune System

Significant development of the immune system occurs peri-natally [132]. Therefore, it is possible that *in utero* programming may have an effect on the immune system that is seen in later life, due to changes induced in the structure and function of immune cells. One such factor that may induce programming are the type of fatty acids available to the foetus during development and foetal growth.

1.6.1.1. Programming of the Immune System by Fatty Acids

Fatty acids are known to affect the immune system (see 1.4), but whether they can affect the immune system during development of the foetus, and if any effects last into adulthood is less clear. It would be sensible to assume they are likely to because of their roles in gene regulation and their effects in influencing the development and maturation of the immune system. Research carried out in the University of Southampton suggests that altering the intake of *n-3* and *n-6* fatty acids in pregnancy can affect the fatty acid composition and function of immune cells of rat foetuses [133]. However, as cells were taken from rats before parturition, it is uncertain whether effects are long-lasting. Olsen *et al.* [134] demonstrated a

reduction in asthma diagnosis in children aged 16 years old whose mothers had consumed fish oil capsules (2.7 g *n-3* PUFAs) compared to those receiving olive oil or no capsules during pregnancy. Studies involving human pregnancies are complicated by the fact that nutritional interventions must occur later in pregnancy than those involving animals, for example, for whom conception can be controlled more easily and therefore interventions may start as soon as the animal is identified as being pregnant, or likely to be so. As such, any effects that may have occurred due to a critical window earlier in pregnancy would be more likely to be missed in studies involving humans. It is also unclear whether fatty acid intake in pregnancy can affect sensitisation to allergens in offspring, which is of particular interest as sensitisation to allergens has been shown to be present at birth, suggesting that the factors that lead to sensitisation are active in the foetus [132, 135], and as such they may be affected by fatty acid intake. Again, whether any effects are irreversible and long-lasting remains to be determined.

1.6.1.2. Programming of the Immune System by Stress

Pre-natal stress can be induced by a number of different methods, and whilst the effect of protein restriction in pregnancy on the immune system has not been looked at directly, the effect of other types of pre-natal stress on the immune system have been. In rats, environmental stress in pregnancy has been shown to reduce T- and B-cell proliferation in offspring [136], and levels of IgG in serum [137], whilst social stress has been shown to reduce abundance of T_H cells in the offspring [138]. More recently, an effect on the second generation of diet from the F0 generation has been discovered [139]. There are no systematic studies of the effect of this maternal protein restriction on immune function in the offspring in the next or subsequent generations.

1.7. Summary, Hypotheses and Objectives

Research to date has suggested that fatty acids may affect the immune system, through their (and those of their metabolites) effects on membranes, mediators, and gene expression. However, despite the research carried out, there still exist gaps of knowledge where further research is required. In addition, programming by fatty acids may affect the immune system, due to induction of stress and/or structural and functional changes to immune cells, but whether this effect is long-lasting is uncertain. In order to address some of these gaps in knowledge of the effect of programming and of fatty acids on the immune system, the experiments detailed in the following chapters were carried out. These experiments aimed to examine the effects of fatty acids and / or programming of foetal physiology on different aspects and at different stages of development of the immune system, and functional consequences of these changes, in models of clinical relevance.

1.7.1. Effect of Dietary Fatty Acids on the Murine Immune Response using Vaccination as an Immune Challenge (Chapter 2)

The study described in Chapter 2 investigates the effect of diets differing in fatty acid composition on the recall response in a mouse model of vaccination, using Influvac (an influenza vaccine). The study set out to test the hypothesis that altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing an increased or decreased recall immune response to Influvac.

1.7.2. The Effect of Dietary Fatty Acids on a Mouse Model of Allergic Sensitisation (Chapter 3)

The study described in Chapter 3 aimed to determine the effect of diets differing in fatty acid composition on the immune response in a mouse model of allergy to the egg protein ovalbumin. It set out to test the hypothesis that altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing increased or decreased susceptibility to the allergen to which the mice had been sensitised.

1.7.3. Effect of Fatty Acids in Pregnancy on Markers of Immune Function in the Offspring (Chapter 4)

The study described in Chapter 4 aimed to investigate the longevity of any effect of maternal dietary fatty acids in rat pregnancy on markers of immune status, in particular the types of T- and B-lymphocytes. It set out to test the hypothesis that altering maternal fatty acid intake can cause structural changes in foetal and / or maternal immune cells, which in turn may cause long-lasting effects on immune function.

1.7.4. Effect of Early Exposure to Fatty Acids on Later Allergic Sensitisation Examined using a Mouse Model (Chapter 5)

The study described in Chapter 5 aimed to determine the effect of diets differing in fatty acid composition, given during gestation and / or lactation, on the immune response in a mouse model of allergy to the egg protein ovalbumin. It was hypothesized that altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing them to become more or less susceptible to their allergy. In addition, mice may be more susceptible to the effects of fatty acids during gestation than lactation, if this is a critical window of fatty acid effect on immune system development.

1.7.5. Effect of a Low Protein Diet in Pregnancy on the Immune System of Offspring in Two Generations (Chapter 6)

The study described in Chapter 6 aimed to determine the effect on the immune system over two generations of diets consumed during rat pregnancy that provided adequate or low protein. It was hypothesized that protein restriction in pregnancy would cause changes in lymphoid tissue cell abundance and weight, and that changes observed in the F1 generation would be seen in the F2 generation.

Chapter 2. Effect of Dietary Fatty Acids on the Murine Immune Response using Vaccination as an Immune Challenge

2.1. Introduction

Host defence against pathogens includes the inflammatory response. Therefore, the widely reported anti-inflammatory effects of long chain *n-3* PUFAs may prove detrimental in situations where an increased immune response is required, for example during viral infection. However the effect of *n*-3 PUFAs on integrated immune responses in vivo is not well described. Therefore a vaccination model was used to evaluate and compare the effects of various fatty acid rich diets on the immune response in vivo. The vaccination was with the seasonal influenza vaccine. This model was chosen as it has the benefit of inducing an immune response to a virus, without inducing the symptoms of infection. In addition, the use of an *in vivo* model has the advantage that the immune response as a whole can be examined. This offers an advantage over studies that have evaluated individual components of the response separately and by studying cells *ex vivo*. The responses and effects seen in such studies may not reflect the in vivo situation and may not reflect the entire integrated response. The study was carried out in collaboration with the University of Utrecht where the model had been established. The author was involved in tissue and data collection on the day of euthanisation, whilst Dr. Astrid Hogenkamp and Dr. Naomi van Vlies at the University of Utrecht carried out feeding and experiments prior to this. The author analysed all data produced.

2.1.1. Influenza

The influenza ("flu") virus is classified into three strains (A, B, and C) based on antigenic differences in two of the internal proteins, nucleoprotein and matrix protein [140]. Strains B and C are predominately found in humans, whilst strain A can infect several species, and even cross species barriers [141]. Strains A and B are the two types which commonly cause disease in humans [140]. Strain A is further categorised based on haemagglutinin (H) and neuraminidase (N) surface antigens, with fifteen subtypes of H and nine subtypes of N identified [140]; the 2009 flu pandemic was caused by the virus influenza A (H1N1), commonly referred to as "Swine flu" [142].

The influenza virus infects the cells of the upper respiratory system, and is characterised by fevers, and chills, often with accompanying cough, sore throat, head ache and muscular pain [143]. During infection, the influenza virus binds to sialic acid-containing cell surface glycoproteins. The virus is endocytosed, and the low pH of the endosomes causes virus and endosome membranes to fuse. This causes the release and import into the nucleus of viral nucleocapsids, and subsequently mRNA synthesis by viral polymerases.

Viral mRNAs are transported to the nucleus, and undergo viral mRNA replication and secondary mRNA synthesis. The result of this is a second round of synthesis of viral RNA and proteins, encapsulation and export from the nucleus of viral nucleocapsids, and budding from the plasma membranes of mature virions. This is summarised in Figure 2-1.

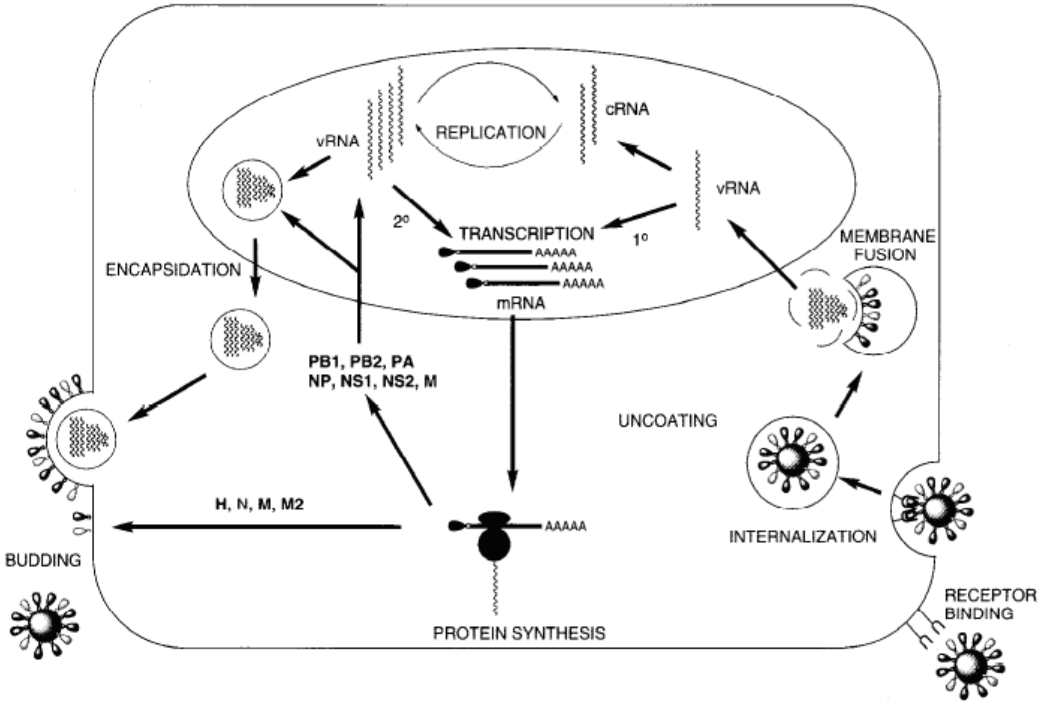

Figure 2-1: Infection of cells by the influenza virus, and subsequent viral replication. The influenza virus is endocytosed, and fuses with the endosome membrane. Viral mRNA is replicated, and secondary mRNA replication occurs. Viral mRNA and proteins are encapsulated, and mature virions bud from the plasma membrane. H, haemagglutinin; M, matrix protein; M2, M2 channel protein; N, neuraminidase; NP, nucleoprotein; NS1, NS2, proteins not found in mature virus; PB1, PB2, PA, polymerase complex. Adapted from Meanwell and Krystal [144].

Cells act to limit spread of the virus through innate and adaptive immune responses; via transcription factor systems, which result in the production of chemokines and cytokines, antigen presentation by APCs, and activation of apoptotic pathways [143]. These are summarised in Figure 2-2.

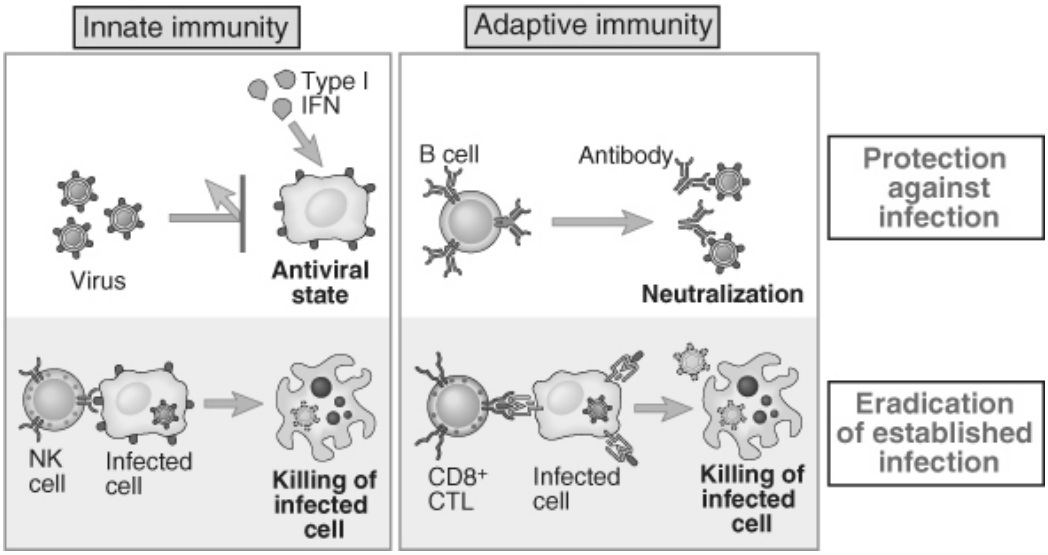

Figure 2-2: Innate and adaptive immune responses to viruses. Innate immunity is mediated by type I IFNs, which prevent infection, and NK cells which kill infected cells. Adaptive immunity is mediated by antibodies, which neutralise infection, and CTLs, which kill infected cells. Figure taken from Abbas and Lichtman [5].

The exact chemokines produced vary depending on the type of cell infected [143]. However, the end result is the same – recruitment of immune cells to the site of infection. Cytokines meanwhile, activate the host anti-viral defence systems. The main cytokines produced are type I IFNs (α and β). These act to inhibit viral replication in infected and uninfected cells by inducing an "anti-viral state" [5]. This is a term used for a cell which has reacted to type I IFN and is resistant to viral infection, because type I IFN has induced production of several enzymes, such as 2′, 5′ oligoadenylate synthase, which affects transcription of viral RNA and DNA, and viral replication. Type I IFNs increase the development of T_H1 cells in humans, as well as the expression of class I MHC molecules, which are recognised by CTLs, thereby increasing recognition of virus infected cells and efficiency of CTLs [5]. NK cells kill infected cells, including those in which the virus has prevented class I MHC expression. NK cells also respond to IL-12, produced by macrophages, and produce IFN-γ, which in turn activates macrophages to destroy phagocytosed microbes. NK cells, in common with CTLs, have granules that contain perforin, a protein which creates pores in target cells. They also contain granzymes, enzymes that pass through the membranes of target cells, and induce apoptosis. By inducing apoptosis of influenza virus-infected cells, NK cells (and CTLs) eliminate established infection. NK cells are an important defence mechanism against viruses during the early stages of infection [5].

Antiviral antibodies function to prevent virus attachment and entry into cells. This is achieved through attachment to the viral envelope or capsid antigens, opsonisation of viral particles and promotion of their clearance by phagocytes; activation of the

complement system is also involved, and acts to promote phagocytosis. Antibodies are able to neutralise extracellular viruses, but are unable to unable to enter cells, and therefore cannot act once the virus penetrates cells and start to replicate. This instead, is the role of CTLs, which act on cells containing intracellular viruses. Cells that are not APCs, such as tissue cells, may be phagocytosed by APCs, in particular dendritic cells, to allow antigen presentation, in a process known as "cross-presentation". Presentation of antigen to naïve CD8+ cells, results in activation and proliferation of CTLs, and induction of cell death of infected cells.

2.1.2. Dietary Fatty Acids and Influenza

Section 1.4 reviewed studies examining the influence of fatty acids on different aspects of immune function or the immune response. These studies have revealed that amongst the different fats, oils and fatty acids tested, the long chain *n-3* fatty acids EPA and DHA exert the greatest effect, although not all findings are consistent. Most often an impairment in immune cell function is caused by these fatty acids. Such an impairment would not be desirable when it is necessary to mount an immune response e.g. during vaccination or infection. However the effect of different fats, oils and fatty acids on responses to vaccination or infection are not well investigated. Furthermore the exact effects of *n-3* fatty acids are often unclear because comparisons have been made to different "controls" in different studies. Finally, many studies in experimental animals have used extremely high fat diets, which are not usually consumed by those animals. Therefore there is a need to evaluate long chain *n-3* fatty acids in comparison to common saturated and *n-6* fatty acids and shorter chain *n-3* fatty acids in suitable models of immune challenge. Vaccination and subsequent DTH response to the vaccine present a useful model for such a comparison. A DTH response is shown in Figure 2-3.

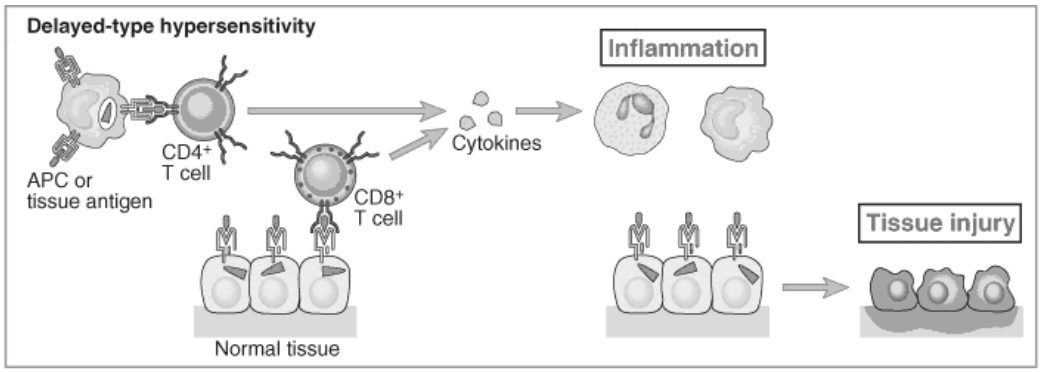

Figure 2-3: In delayed-type hypersensitivity reactions, CD4+ cells, and sometimes CD8+ cells, secrete cytokines in response to antigens, which stimulate cytokine production and phagocyte activation, causing inflammation and tissue injury. APC, antigen presenting cell. Taken from Abbas and Lichtman [5].

Although many studies have demonstrated a suppressive effect of long chain *n-3* fatty acids on various aspects of immune function, these observations have often been seen against a very high fat background and they are counter to the anticipated action of these fatty acids. Prostaglandin (PG) E_2 derived from the *n-6* fatty acid arachidonic acid has been shown to impair T cell function and the production of key cytokines like IL-2 and IFN-γ [145]. Since long chain *n-3* fatty acids reduce PGE_2 production [105], they should enhance T cell function and improve the vaccination and DTH responses.

Whilst no studies to date have examined the effect of dietary fatty acids on vaccination with an influenza vaccine in rodents, several studies have exposed mice to the influenza virus by inhalation. Schwerbrock *et al.* [146] infected male BALB/C mice with the influenza virus following two weeks of feeding diets containing corn oil (5 g/100 g diet) or a combination of fish and corn oil (4 g fish oil plus 1 g corn oil/100 g diet). They demonstrated a reduced lung inflammation in fish oil fed mice, but also a higher mortality rate, higher lung viral load 7 days after infection, and longer recovery period following infection. Splenic NK cell activity was lower in fish oil than corn oil fed mice, but there was no difference in lung NK cell activity. The authors also reported a reduction in $CD8^+$ T-cells in lungs of fish oil fed mice. Byleveld *et al.* [147] infected BALB/C mice after feeding diets containing sunflower oil (3 g/100 g diet) in combination with 17 g of either fish oil or tallow/100 g diet for fourteen days. Fish oil fed mice had higher virus titres 1 and 5 days after infection, reduced lung IFN-γ production, and serum IgG and IgA specific antibodies. No difference was seen in IFN-α / β production in lungs, or ratio of $CD4^+$ to $CD8^+$ T-cells in the bronchial lymph node, between dietary groups. The authors interpreted these results as the fish oil fed mice showing reduced viral clearance, associated with reduced IFN-γ and immunoglobulin production, compared to the tallow group. A later study by the same group, using the same experimental procedures also showed reduced

CTL cytotoxicity in the fish oil group, despite the increased splenic lymphocyte proliferation in response to the virus compared to the tallow group [148].

The influenza immunization model has been used to examine the effect of dietary fatty acids on immune response in other species. Newborn piglets receiving 0.63 % arachidonic acid (AA) and 0.34 % docosahexaenoic acid (DHA) in a milk formula for thirty days, had reduced influenza-specific CD4+ and CD8+ *ex vivo* proliferative responses, and increased IL-10 production compared to animals receiving sows milk or unsupplemented milk formula, following immunization [149]. Kelley *et al.* [101] found an increased proliferation response to the influenza virus following immunisation in adult males receiving 1.5 g AA/day for 130 days. The differences between the two studies may be explained by AA supplementation in combination with DHA having a different effect from sole supplementation with AA, level of supplementation, and differences in length of supplementation period, species used and age of subjects.

2.1.3. Mouse Model of Vaccination

Vaccination with killed or attenuated pathogens provides a means of examining the *in vivo* immune response to the pathogen, without inducing symptoms of the disease that live pathogens would do [106]. Repeat vaccination can be used to examine recall responses (DTH) to the immune challenge. Influvac is a vaccine that contains an attenuated form of the influenza virus. By feeding mice diets containing different fatty acids prior to vaccination, it is possible to examine the effect of dietary fatty acids on the immune system in an *in vivo* model of immune response. C57BL6 mice were used as they have a T_H1 dominant immune response [150].

2.1.4. Aims of the Study

This study aimed to determine the effect of diets differing in fatty acid composition, including different levels and different types of *n-3* and *n-6* fatty acids, on the immune response in a mouse model of vaccination using Influvac. Altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing them to become more or less able to respond to the influenza virus.

In order to achieve this aim, six diets were fed to mice from 3 to 4 weeks of age until 8 to 9 weeks of age. One diet used salmon oil as the fat source; this diet is high in long chain *n-3* fatty acids (EPA plus DHA). The second diet used soybean oil as the fat

source; this diet contains a balance of plant *n-3* and *n-6* fatty acids. The third diet used sunflower oil as the fat source; this diet is rich in the *n-6* fatty acid linoleic acid. The fourth diet used linseed oil as the fat source; this diet is rich in the short chain plant *n-3* fatty acid α-linolenic acid (ALA). The fifth diet used tallow as the fat source; this diet is rich in saturated fatty acids. These five experimental diets were high in fat (13 % by weight). The final diet used was standard chow, a low fat (3 % by weight of the diet) diet whose fat source is soybean oil.

2.1.5. Hypotheses

This study set out to test the following hypotheses:

- Feeding the salmon oil diet will increase the immune response to Influvac.
- Feeding the sunflower oil diet will reduce the immune response to Influvac.
- Feeding the beef tallow diet will reduce the immune response to Influvac.
- Feeding the linseed oil diet will increase the immune response to Influvac compared to the sunflower oil diet, as ALA can be converted to EPA, but will be less effective than the salmon oil diet, as fatty acid metabolism is a less effective way of producing EPA than direct provision in the diet.

2.2. Materials and Methods

2.2.1. Animals, Diets and Materials

2.2.1.1. Animals

3 to 4 week old male C57BL6 mice were obtained from Charles River (Maastricht, The Netherlands). Animals were housed in groups of four in shoebox cages, and kept at 22°C ± 2°C, under a 12 hour light: 12 hour dark cycle in temperature and light-controlled room in the animal facility at the Pharmacology Department, University of Utrecht. Animals had *ad libitum* access to tap water and 150 g food was provided on alternate days. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

2.2.1.2. Diets

The high-fat (130 g fat/kg diet) experimental diets (sunflower oil, salmon oil, soybean oil, linseed oil and tallow) and low-fat (30 g fat/kg diet) soybean oil-based chow (CRM-1) were obtained from Special Diet Services (Witham, Essex, UK). Experimental diets were produced by replacing 10 % of the wheat content of CRM (P) (diet formulated by Special Diet Services) with either sunflower oil, salmon oil, soybean oil, linseed oil or tallow and vitamin E levels were standardised to 181 IU/kg diet. The nutrient composition of diets is shown in Appendix One. The fatty acid composition of each diet is described in Section 2.3.1.

2.2.1.3. Materials

Influvac (2005/2006) was from Solvay Pharmaceuticals B.V. (Weesp, The Netherlands). Stimune was from Cedi-diagnostics, Lelystad, The Netherlands. Phosphate buffered saline (PBS) was from BioWhittaker, Verviers, Belgium. Sandwich ELISAs were from PharMingen (San Diego, CA, USA). RPMI-1640 was from Roche Applied Science (Burgess Hill, UK). RNA-later was from Qiagen (Crawley, UK). Foetal calf serum (FCS), penicillin and streptomycin were from Life Technologies (Gaithersburg, MD, USA). Antibodies were from AbD Serotec (Oxford, UK). FACS buffer was prepared by adding 1% BSA (Roche Diagnostics, GmbH, Mannheim, Germany) to PBS. Fix / perm buffer and permeabilisation buffer were from EBioscience (San Diego, CA). Diff-Quick was from Dade AG (Düdingen, Switzerland).

2.2.2. Methods

An overview of the study design is shown in Figure 2-4. Forty-eight male C57BL6 mice were randomised to receive one of the six diets (n = 8/diet) fourteen days before vaccination (at day -14). Vaccination was carried out at days 0 and 14. Delayed-type hypersensitivity (DTH) tests were carried out on days 10 and 11. Euthanisation occurred by *intra peritoneal* injection of 1 ml 10 % urethane on day 23 ± 1.

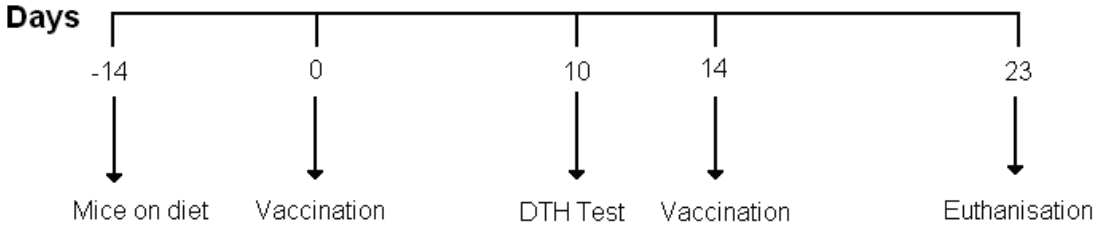

Figure 2-4: Schematic diagram of experiment examining the effect of dietary fatty acids on a vaccination mouse model. DTH, Delayed-Type Hypersensitivity.

2.2.2.1. Vaccination of Mice with Influvac

Mice were vaccinated with Influvac at days 0 and 14. 100 µl of Influvac was added to 4.9 ml PBS and 5 ml of the adjuvant Stimune. Mice received of 100 µl of this solution by subcutaneous injection.

2.2.2.2. Determination of DTH Response to Influvac

DTH response was measured on day 10 and 11. This involved intracutaneous injection of Influvac into both ears. Duplicate measurements of ear thickness were made bilaterally with an engineer's micrometer (Mitotoyo, Veenendaal, Netherlands) before injection and 24 h after Influvac injection.

2.2.2.3. Fatty Acid Analysis of Diet and Erythrocytes

At euthanisation, blood was collected by cardiac puncture, transferred into EDTA tubes and stored on ice. Blood samples were centrifuged at 14000 rpm for five minutes, and plasma removed for antibody analysis (see 2.2.2.4). Erythrocytes were washed with PBS containing 5 mM EDTA and then centrifuged at 14000 rpm for five minutes. This step was repeated a further two times. Erythrocytes were then resuspended in an equal volume of PBS and EDTA and stored at -80 °C until analysis. Erythrocytes and samples of diet were analysed for fatty acids. Samples of plasma were adjusted to 0.8 ml with 0.9 % NaCl, if 0.8 ml sample volume of plasma was not available. 50 µg of 15:0 (Dipentadecanoyl PC) was added as an internal standard. 5 ml of chloroform : methanol

(2:1 v/v) , which contained BHT (50 mg/l) as antioxidant, and then 1 ml 1 M NaCl were added to samples before vortexing. Samples were centrifuged at 2000 rpm for 10 minutes with a low brake. The lower phase was collected by aspiration and then dried under nitrogen at 40 °C.

Fatty acid methyl esters were prepared by adding 0.5 ml dry toluene to samples, before vortexing. 1 ml methanol containing 2 % (v/v) H_2SO_4 was added and then gently mixed. Samples were heated at 50 °C for 2 hours. After cooling, 1 ml of neutralising solution (0.25 M $KHCO_3$ (25.03 g/l), 0.5M K_2CO_3 (69.1 g/l)) was added before the addition of 1 ml dry hexane. Samples were centrifuged at 1000 rpm for 2 minutes with low brake. The upper phase, which contains the fatty acid methyl esters, was collected and then dried under nitrogen at 40 °C. 1 ml dry hexane was added to samples and vortexed, before being transferred to a gas chromatography (GC) vial. 2 µl of sample was injected by a Hamilton syringe into an HP6890 series GC system (Hewlett Packard) fitted with a 30 m x 32 mm BPX70 capillary column, with film thickness of 0.25 µm. The carrier gas was helium at a rate of 2.0 ml / min, and the split / splitless injector was used at a ratio of 10:1. The temperature profile was as follows: initial temperature 115°C, hold 2 minutes, ramp 10°C/minutes to 200°C, hold 10 minutes, ramp 60°C/minutes to 245°C, and hold 4 minutes Separation was recorded and analysed with GC Chemstation (Agilent technologies) software. Fatty acid methyl esters were identified by comparison with standards run previously.

2.2.2.4. Analysis of Plasma Immunoglobulin Concentrations

Plasma concentrations of IgG1 and IgG2a were determined using sandwich ELISAs, following the manufacturer's instructions.

2.2.2.5. Analysis of Immune Tissue, Fat and Liver

Spleen, thymus, epididymal fat pads and liver were removed and weighed. Bone marrow cells were collected by cutting between the metaphysis and diaphysis at both ends of both femurs, and flushing out bone marrow using a 21G x 1 1/2" needle and 10 ml syringe containing PBS.

2.2.2.6. Histological Analysis of the Thymus

For histological analysis, part of the thymus was stored in formaldehyde (10 % neutral buffered formalin) for at least 24 h before being processed using a LEICA TP1020

Automatic Tissue Processor. Tissues were embedded in paraffin using a LEICA EG 1150c. 5 μm sections were cut, and stained with haematoxylin/eosin. Cortex/medulla ratio was determined by examining the surface area of the cortex and medulla using an Olympus BX50 microscope (Zoeterwoude, The Netherlands) and Image Pro Plus software (IPWIN32; from Media Cybernetics, Silver Spring, MD, USA).

2.2.2.7. Determination of Cytokine Production by Splenocytes

IFN-γ, IL-4 and IL-10 production by spleen cells in response to Influvac was analysed. A single cell suspension was prepared by mashing cells through 70 μm nylon cell strainers (BD Biosciences, Franklin Lakes, NJ, USA) with 10 ml PBS. Cells were counted using a Bürker-Türk counting-chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany) and 8 million cells removed for FACS analysis (see 1.2.2.11). The remainder of the cells were collected by centrifugation at 1300 rpm for five minutes, and resuspended in RPMI containing 1% penicillin/streptomycin, 10 % FCS, and 20 μM β-mercaptoethanol. 8 x 10^5 spleen cells per well were cultured in 96-well round-bottom plates (Greiner Bio-One, Kremsmuenster, Austria) in the presence or absence of 0.1 μg/ml Influvac. Cells were cultured for 5 days at 37 °C in 5 % CO_2. Supernatants were removed and stored at -80 °C prior to ELISA analysis. The lower detection limits of the ELISAs were 16 pg/mL for IFN-γ, 16 pg/mL for IL-4 and 63 pg/mL for IL-10.

2.2.2.8. Determination of Cell Subsets in Lymphoid Tissues using Flow Cytometry

Flow cytometry (FACs) uses light to classify cells based on their differences in light scatter and fluorescence. When light hits the cells, it is scattered in either a forward or sideways direction. Forward scatter (FSC) is related to the size of the cell, whilst side scatter (SSC) depends on the granular content of the cell. Although FSC and SSC are unique for every cell, the same cell types (i.e. lymphocytes, granulocytes, monocytes) produce similar FSC and SSC measurements, and therefore can be clustered on a dot plot (cytogram) of SSC against FSC, as shown in Figure 2-5. Thus, cell types can be identified from a heterogeneous sample.

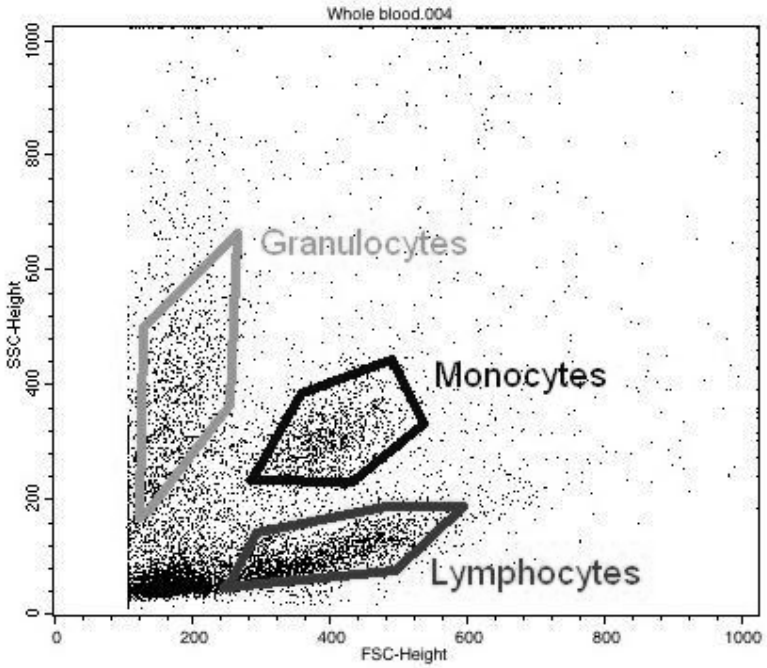

Figure 2-5: Cytogram of lysed whole blood analysed by flow cytometry, showing how types of cells can be identified by differences in forward scatter (FSC, measures size of the cell) and side scatter (SSC, measures granular content of cell). Thus, cells of interest can be selected by gating – the bottom gate shows lymphocytes, the middle gate shows monocytes, and the top gate shows granulocytes.

Once the cell type of interest has been selected by gating, cells can be further analysed for properties such as the expression of cell surface proteins, provided they have been stained with a fluorescent probe. A typical quadrant plot (in this case for lymph node cells stained with antibodies to CD3 and CD8) is shown in Figure 2-6.

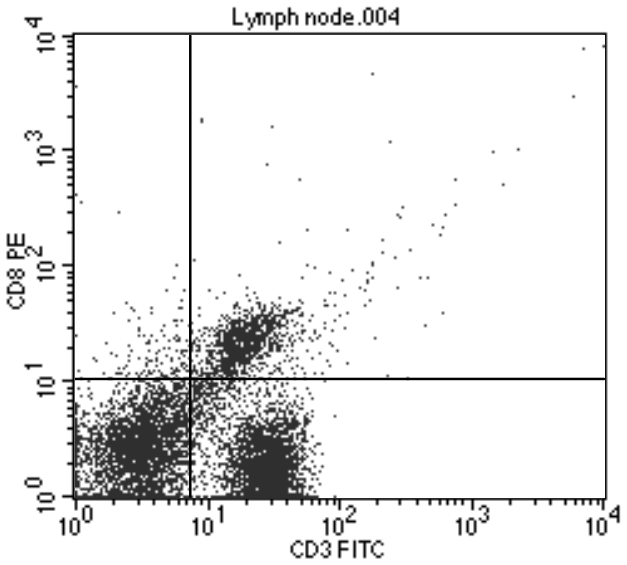

Figure 2-6: Quadrant plot showing typical results from analysis by flow cytometry. CD3 is measured along the horizontal axis and CD8 along the vertical axis. The horizontal and vertical black lines show where boundaries lie for determining whether cells are positive (above or to the right of the line) or not for these proteins. Therefore, a dot in the top left quadrant represents a CD3-CD8+ cell, in the top right quadrant a dot represents a CD3+CD8+ cell, in the bottom left cells are CD3-CD8- and in the bottom right quadrant appears cells that are CD3+CD8-.

The number of cells in each quadrant can be used to determine the proportion of cells expressing each CD protein. For example, in Figure 2-6, cells in the top left quadrant are $CD3^-CD8^+$, those in the top right are $CD3^+CD8^+$, bottom left shows $CD3^-CD8^-$, and bottom right cells are $CD3^+CD8^-$.

In flow cytometry, measurements are made rapidly and separately on each cell in turn, rather than producing averages of the sample as a whole. It also has the advantage that more than one stain can be used on the same sample to provide multiple measurements, as was the case in Figure 2-6, where two stains were used (anti-CD3 and anti-CD8).

Single cell suspensions of thymus and bone marrow were made as for spleen cells 2.2.2.7), although blocking buffer (FACS buffer + 5 % FCS) was used instead of PBS. Cells were counted (2.2.2.8), and 8 x 10^6 spleen cells, 2 x 10^6 thymus cells, and 1 x 10^6 bone marrow cells added to 2 ml blocking buffer. After thirty minutes, cells were collected by centrifugation at 1300 rpm for five minutes, and resuspended in FACS buffer (PBS containing 1 % BSA). 2 x 10^5 cells were added to a 96-well round-bottom plate that contained 50 µl antibody and left in the dark at room temperature for 60 minutes. For CD4/CD25/FoxP3, twice as many cells were added to each well. Antibodies used are listed in Table 2-1.

Table 2-1: Antibodies used for FACS analysis

Antibody	Cell type identified	Spleen	Thymus	Bone marrow
Anti-CD3/anti-CD4/anti-CD8	T-cells, T_H cells, CTLs	X	X	
Anti-CD3/anti-CD4/anti-CD62	L-selectin expressing T_H cells	X	X	
Anti-CD3/antiCD4/anti-CD69	Activated T_H cells	X	X	
Anti-CD19	B-cells	X		X
Anti-F4-80	Macrophages	X		
Anti-CD4/anti-CD25/anti-FoxP3	T_{REG} cells	X	X	

2.2.2.8.1. Analysis with antibodies excluding CD4/CD25/FoxP3

For all wells except the CD4/CD25/FoxP3, plates were centrifuged at 1300 rpm for five minutes, and supernatants discarded. Cells were resuspended in 150 µl FACS buffer, and re-centrifuged at 1300 rpm for five minutes, and supernatants discarded. This step was repeated twice more, but using only 100 µl FACS buffer. Cells were resuspended with 100 µl FACS buffer, and analysed using a Becton Dickinson FACSCalibur flow cytometer (Breda, The Netherlands) and FCS Express version 3 software (De Novo Software, Los Angeles, CA, USA), which recorded 10000 events per sample.

2.2.2.8.2. Analysis with CD4/CD25/FoxP3 Antibodies

CD4/CD25/FoxP3, plates were centrifuged at 1300 rpm for five minutes, and supernatants discarded. Cells were resuspended in 150 µl FACS buffer, centrifuged at 1000 rpm for five minutes and supernatants discarded. Cells were resuspended in 200 µl Fix/Perm buffer, and incubated for thirty minutes at 4 °C. Plates were centrifuged at 1800 rpm for ten minutes, the supernatant discarded, and 150 µl permeabilisation buffer added. This was left for ten minutes in the dark, before being centrifuged at 1800 rpm for ten minutes, and the supernatant discarded. This step was repeated. Cells were resuspended in100 µl blocking buffer, and plates left in the dark for fifteen minutes. 10 µl anti-FoxP3 was added to each well, and plates incubated for thirty minutes at 4 °C. Plates were centrifuged at 1800 rpm for ten minutes and supernatant discarded. Cells were washed twice with FACS buffer, resuspended in FACS buffer, and analysed as for plates in 2.2.2.8.1.

2.2.2.9. Statistical Analysis

Results were analysed using SPSS version 15.0. Parameters were analysed using Levene's test of homogeneity to ensure groups had equal variances, in which case they were compared using one-way analysis of variance (ANOVA), followed by *post-hoc* testing (Tukey). If Levene's test showed unequal variances to be present, groups were compared using the Kruskal-Wallis test. Where this was used, it is reported in the results section. If the Kruskal-Wallis test indicated a significant effect, Mann-Whitney U independent group comparisons tests were then carried out to compare each combination of groups.

2.3. Results

2.3.1. Fatty Acid Content of the Different Diets

Fatty acid composition of diets is shown in Appendix One. Abundance of fatty acids of interest is shown in Figure 2-7.

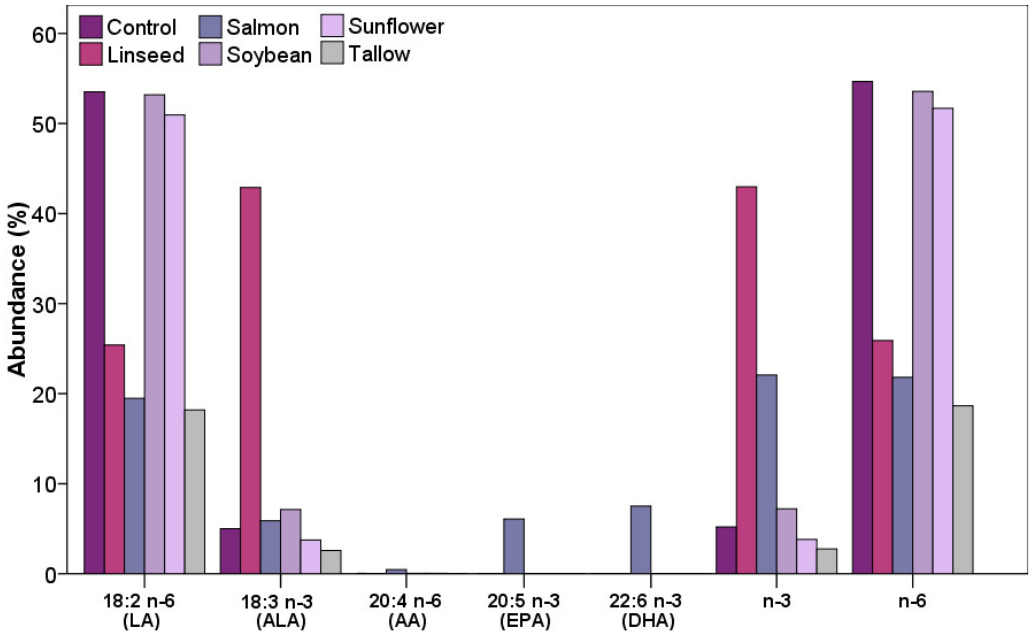

Figure 2-7: Profile of individual and total *n-3* and *n-6* PUFAs in the diets used

The control (chow) and soybean oil diets had similar profiles of fatty acids, but the total amounts of fatty acids were different: the control diet was formulated to contain a lower percentage (3 %) of fat than the high fat (13 %) experimental diets. The linseed oil diet was rich in the short chain *n-3* fatty acid ALA. The salmon oil diet was rich in the long chain *n-3* fatty acids EPA and DHA. The sunflower oil diet was rich in the *n-6* fatty acids LA. The tallow diet had a low PUFA content, but a similar, if slightly higher *n-3* : *n-6* ratio to the soybean oil diet.

2.3.2. Body weight of Mice fed the Different Diets

Body weight in mice fed the different diets is shown in Figure 2-8.

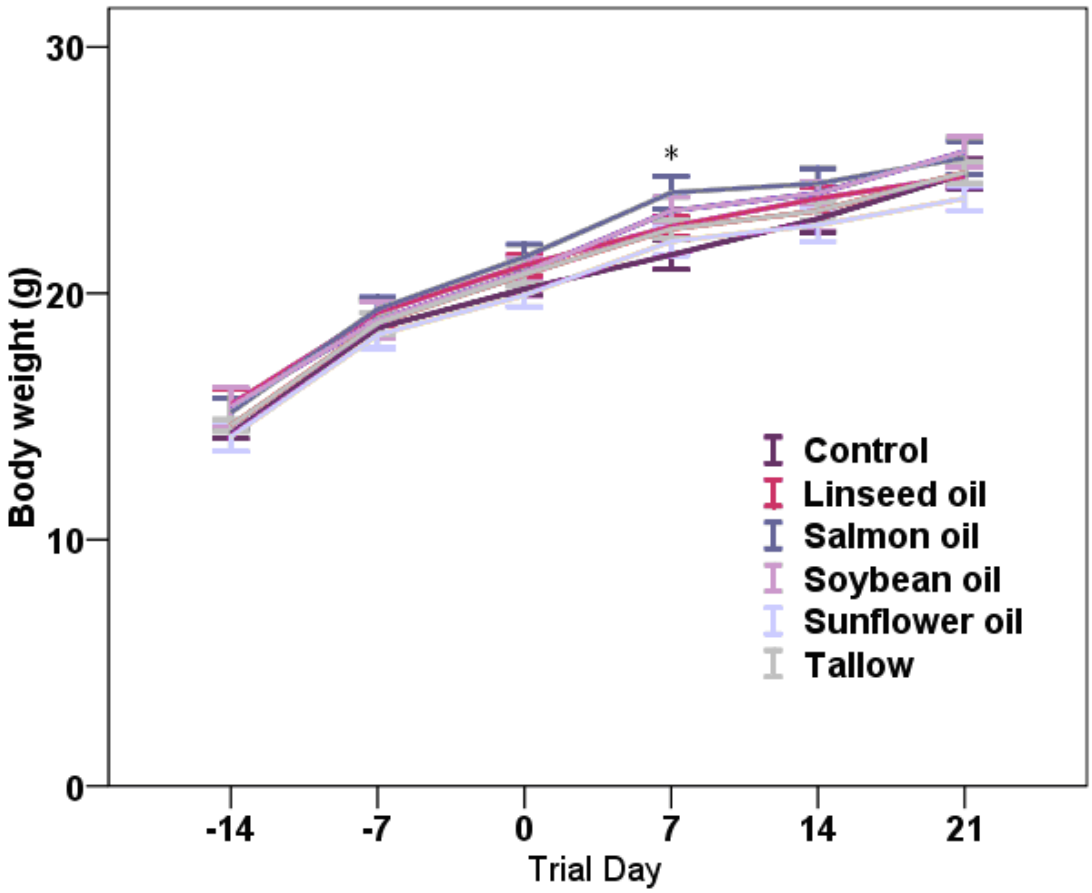

Figure 2-8: Body weight of mice fed the different diets. Data are mean ± SEM. * denotes differences between body weights at different time points.

At day 7, mice in the salmon oil group were heavier than those in the control group. At all other time points, including the day of tissue collection (day 23; data not shown) there was no difference in body weight between dietary groups.

2.3.3. Tissue Weights of Mice fed the Different Diets

Tissue weights of mice fed the different diets are shown in Table 2-2. Liver weight was lowest in the salmon oil group. Animals receiving sunflower oil or tallow diets had heavier livers compared to those receiving the soybean oil diets. Where liver weight was expressed proportionally to body weight, there were no significant differences between dietary groups. There were also no differences between dietary groups in the absolute or relative weight of any other tissue measured, including spleen and thymus.

Table 2-2: Tissue weights of mice fed the different diets. Fat signifies epididymal fat. Data are mean + SEM. Values different letters are significantly different. BW, body weight.

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
Spleen (g)	0.13 ± 0.02	0.09 ± 0.01	0.09 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.11 ± 0.01
Spleen (% BW)	0.49 ± 0.06	0.39 ± 0.02	0.37 ± 0.01	0.39 ± 0.01	0.45 ± 0.02	0.42 ± 0.03
Thymus (g)	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Thymus (% BW)	0.22 ± 0.05	0.21 ± 0.02	0.20 ± 0.02	0.21 ± 0.01	0.21 ± 0.02	0.21 ± 0.01
Liver (g)	1.39 ± 0.12^{bc}	1.26 ± 0.04^{bc}	1.14 ± 0.03^{a}	1.25 ± 0.02^{b}	1.33 ± 0.01^{c}	1.33 ± 0.03^{c}
Liver (% BW)	5.55 ± 0.48	5.19 ± 0.12	4.79 ± 0.07	4.95 ± 0.05	5.19 ± 0.10	5.32 ± 0.12
Fat (g)	0.27 ± 0.03	0.27 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.30 ± 0.02
Fat (% BW)	1.08 ± 0.10	1.11 ± 0.07	1.23 ± 0.10	1.14 ± 0.07	1.12 ± 0.07	1.20 ± 0.08

2.3.4. Fatty Acid Content of Erythrocytes from Mice Fed the Different Diets

The content of fatty acids of interest in erythrocytes from mice fed the different diets is shown in Figure 2-9 (the full data set is shown in Appendix One).

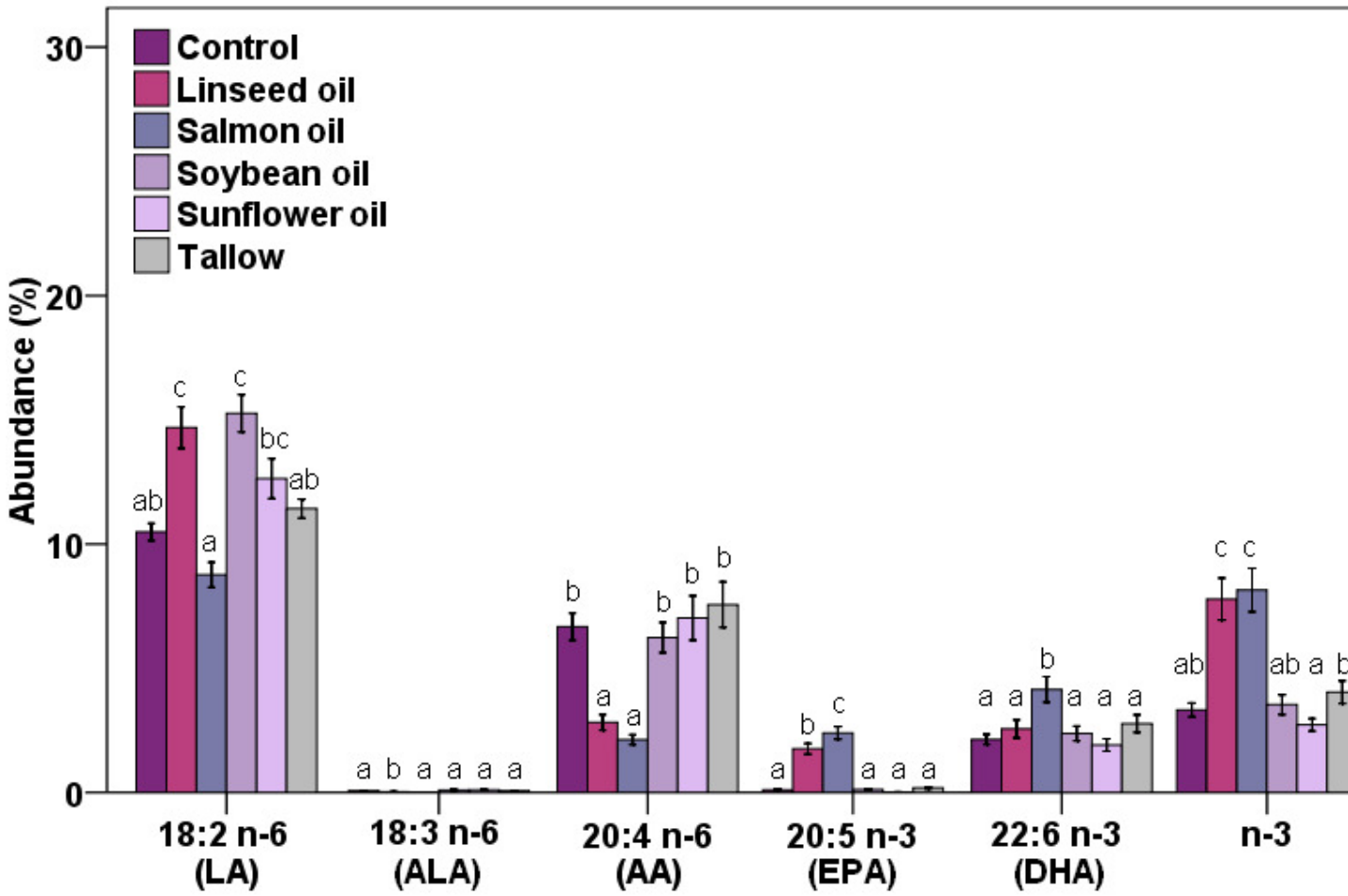

Figure 2-9: Individual and total *n-3* and *n-6* PUFA contents of erythrocytes from mice fed the different diets. Data are mean ± SE denote differences between dietary groups.

LA content was lower in the salmon oil and tallow group than in the linseed and soybean oil groups. The salmon oil group had lower LA content than the sunflower oil group. The linseed oil group had the highest ALA content in erythrocytes. The salmon oil and linseed oil groups had lower AA content than other groups. The salmon oil group had the highest EPA content, followed by the linseed oil dietary group. The salmon oil dietary group had a higher EPA content than the control, linseed oil, soybean oil and sunflower oil groups and a higher DHA content than all other groups. *n-3* PUFA content was lowest in the sunflower oil group, and highest in the salmon oil and linseed oil groups. *n-6* PUFA content was lowest in the salmon oil group.

2.3.5. DTH Response to Influvac in Mice Fed the Different Diets

The effect of diet the DTH response, measured as ear swelling in response to Influvac, is shown in Figure 2-10. Mice in the salmon oil group showed the greatest DTH response and this was greater than in the beef tallow, sunflower oil and linseed oil groups.

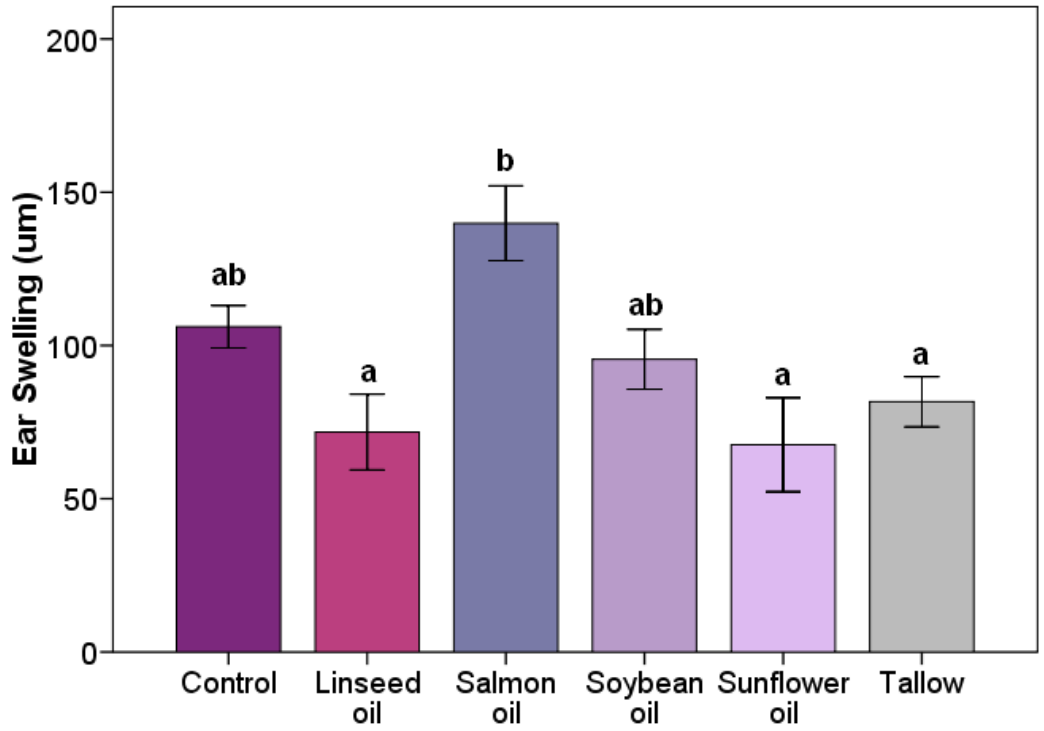

Figure 2-10: Effect of diet on ear swelling in response to Influvac in Influvac-vaccinated mice, measured 24 hours after injection. Data are mean ± SEM. Bars with different letters are significantly different from one another ($p < 0.05$).

2.3.6. Plasma Immunoglobulin Concentrations in Mice Fed the Different Diets

IgG1 levels were below detection limits. IgG2a levels are shown in Figure 2-11. There was no effect of diet on IgG2a levels in plasma, although high fat feeding tended to result in a lower concentration.

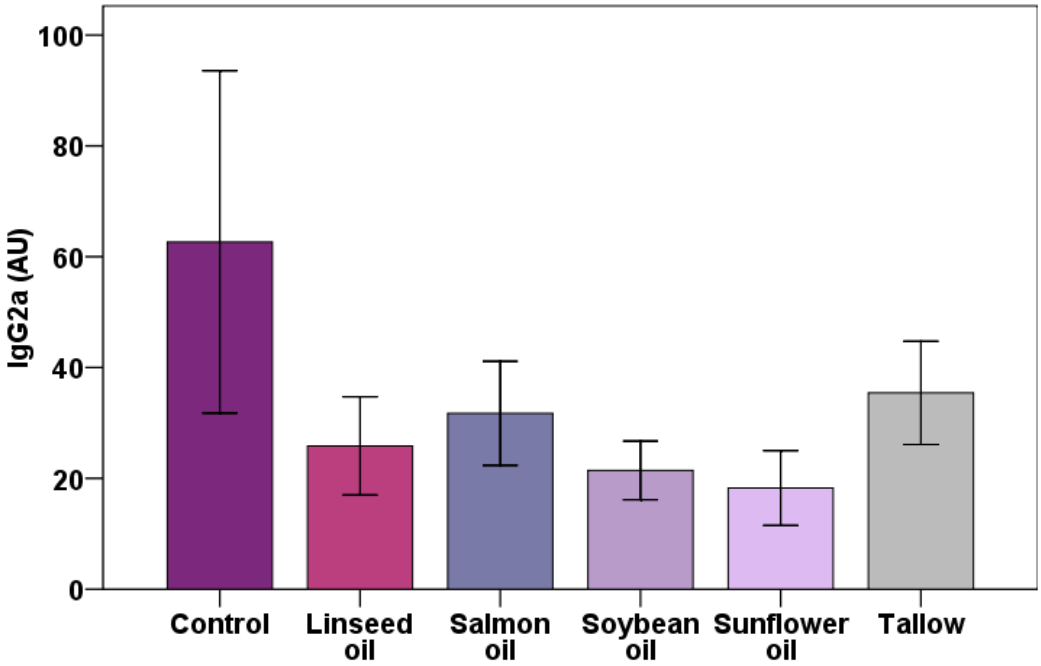

Figure 2-11: Plasma IgG2a concentration in mice fed the different diets. Data are mean ± SEM.

2.3.7. Cytokine Production by Cultured Splenocytes from Mice Fed the Different Diets

IFN-γ, IL-4 and IL-10 concentrations in spleen cell cultures exposed to Influvac were below detectable limits.

2.3.8. Histological Analysis of Thymuses from Mice Fed the Different Diets

The cortex / medulla ratio of thymuses from mice fed the different diets is shown in Figure 2-12. There were no significant differences between dietary groups.

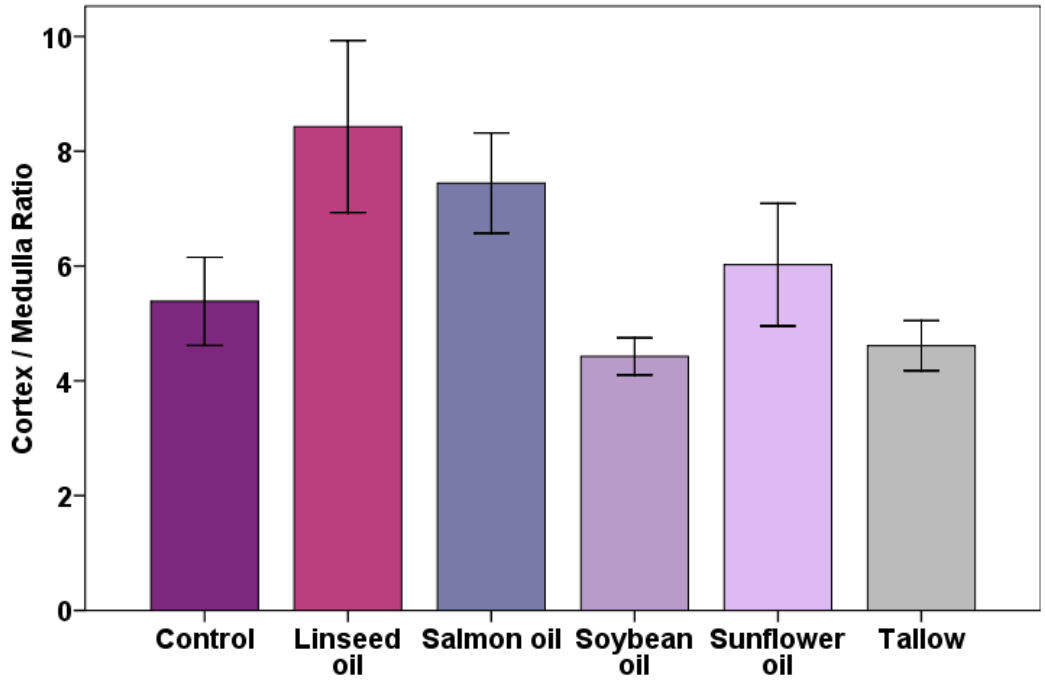

Figure 2-12: Cortex/medulla ratio of thymuses from mice fed the different diets. Data are mean ± SEM.

2.3.9. Cell Subsets in the Thymus, Spleen and Bone Marrow from Mice Fed the Different Diets

Effect of diet on the proportions of different cell types in the spleen, thymus and bone marrow of mice is shown in Table 2-3 to Table 2-5. There was no effect of diet on any of the cell subsets measured.

Table 2-3: Cell subsets within the thymus of mice fed the different diets. Data are mean + SEM.

	Cells Identified	Control	Linseed oil	Salmon oil	Soybean oil	Sunflowe
CD3+	T-cells	73.16 ± 12.48	73.89 ± 12.89	53.27 ± 15.10	72.40 ± 12.82	74.20 ± 13
CD3+ that are also CD4+	T_H cells	90.32 ± 2.41	88.13 ± 2.61	85.83 ± 2.99	90.03 ± 3.11	88.95 ± 2
CD3+ that are also CD8+	CTLs	78.77 ± 5.52	77.78 ± 5.62	70.93 ± 6.09	78.78 ± 5.25	80.61 ± 5
CD3+CD4+ that are also CD62L+	L-selectin expressing T_H cells	70.98 ±0.91	76.68 ± 1.64	77.29 ± 1.44	71.28 ± 1.08	72.43 ± 1
CD4+CD25+	T_{REG} cells	0.41 ± 0.90	1.53 ± 0.14	1.39 ± 0.18	1.06 ± 0.17	1.64 ± 0.
CD4+CD25+ that are also FoxP3+	T_{REG} cells	7.26 ± 2.23	13.45 ± 2.87	10.85 ± 1.64	7.27 ± 1.76	11.11 ± 2

Table 2-4: Cell subsets within the spleen of mice fed the different diets. Data are mean + SEM.

	Cells Identified	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower
F4/80	Macrophages	14.42 ± 1.34	12.18 ± 1.39	11.77 ± 0.37	12.34 ± 1.37	14.51 ± 1.0
CD3+	T-cells	22.07 ± 3.45	21.40 ± 3.14	26.50 ± 1.62	22.74 ± 1.42	23.98 ± 3.6
CD3+ that are also CD4+	T_H cells	56.34 ± 1.79	55.13 ± 4.02	51.28 ± 2.98	54.97 ± 1.97	57.65 ± 3.3
CD3+ that are also CD8+	CTLs	1.80 ± 0.15	1.38 ± 0.19	0.86 ± 0.26	1.53 ± 0.18	1.54 ± 0.2
CD3+CD4+ that are also CD62L+	L-selectin expressing T_H cells	35.13 ± 1.49	41.62 ± 1.59	36.50 ± 2.73	31.76 ± 2.58	42.10 ± 1.0
CD3+CD4+ that are also CD69+	Activated T_H cells	39.30 ± 2.65	35.68 ± 4.80	34.22 ± 2.91	38.14 ± 3.89	37.32 ± 4.8
CD19	B-cells	14.42 ± 1.34	12.18 ± 1.39	11.77 ± 0.37	13.04 ± 1.66	14.51 ± 1.0
CD4+CD25+	T_{REG} cells	2.06 ± 0.47	2.17 ± 0.48	1.70 ± 0.62	2.07 ± 0.47	2.28 ± 0.5
CD4+CD25+ that are also FoxP3+	T_{REG} cells	18.41 ± 2.58	16.66 ± 2.90	14.70 ± 2.30	13.82 ± 2.79	18.02 ± 2.6
CD94+	NK cells	44.21 ± 5.53	41.76 ± 4.28	33.19 ± 4.60	41.82 ± 3.64	46.99 ± 4.0

Table 2-5: B cells within the bone marrow from mice fed the different diets. Data are mean + SEM.

	Cells Identified	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallo
CD19+	B-cells	4.76 ± 0.57	6.26 ± 1.48	7.40 ± 1.96	6.56 ± 1.06	6.99 ± 1.55	6.16 ±

2.4. Discussion

2.4.1. Summary of Findings

This study aimed to determine the effect of diets differing in fatty acid composition, including different types of *n-3* and *n-6* fatty acids, on the immune response in a mouse model of vaccination. This is the first time that this model has been used to investigate the influence of fatty acids on immune function. The study set out to test hypotheses of increased immune response with salmon and linseed oils and reduced immune response with sunflower oil and beef tallow. Using ear swelling in response to Influvac, with which the mice had already been vaccinated, as a measure of DTH, mice receiving salmon oil showed the greatest response while those receiving the tallow, sunflower or linseed oil diets had reduced ear swelling compared to those receiving the salmon oil diet. This suggests the salmon oil diet, and so long chain *n-3* PUFAs, enhance the T_H1 type response. However, examining cytokine and immunoglobulin levels, to determine the effect on T_H1 / T_H2 balance did not produce results supporting these findings. IgG1 levels of plasma and IFN-γ, IL-4 and IL-10 production by spleen cell cultures were below detection limits, and there was no effect of diet on IgG2a levels in plasma.

The fatty acid content of red blood cells generally reflected that of the fatty acids present in diets. At day 7, mice in the salmon group were heavier than those in the control group. At all other time points, including the day of tissue collection there was no difference in body weight between dietary groups. Liver weight was lowest in the salmon oil group. Animals receiving sunflower oil or tallow diets had heavier livers compared to those receiving the soybean oil diets. Where liver weight was expressed proportionally to body weight, there were no significant differences between dietary groups. There were also no differences between dietary groups in the absolute or relative weight of any other tissue measured, including spleen and thymus. There were no significant differences in the cortex / medulla ratio of mice thymuses between dietary groups. There was no effect of diet on the proportions of different cell types in the spleen, thymus and bone marrow.

2.4.2. Fatty Acid Content of Erythrocytes and Diets

In general, the fatty acid content of erythrocytes reflected the fatty acid content of diets. The salmon oil and linseed oil groups had lower AA content of erythrocytes than other groups, whilst the EPA content of erythrocytes was highest in the salmon oil group, and higher in the linseed oil group than in other groups (excluding salmon oil). The salmon oil diet resulted in a higher erythrocyte DHA content than the other diets. These findings

are in agreement with those of other fatty acid feeding studies which report on fatty acid levels in plasma, serum and erythrocytes [70, 71, 79, 151-156].

2.4.3. Tissue and Body Weights

Body weights increased over the period of the trial, in accordance with findings of Poiley [157]. At day 7, mice in the salmon oil group were heavier than those in the control group, but there were no differences at any other time point. This may have been caused by factors such as stress or palatability affecting appetite. However, since food intake was not recorded, and animals were housed in groups of 8, it is impossible to ascertain whether this occurred. Body weights did not differ between dietary groups at the start or the end of the trial, suggesting that this factor is unlikely to have confounded results.

Liver weight was lowest in the salmon oil group. Animals receiving sunflower oil or tallow diets had heavier livers compared to those receiving the soybean oil diets. Where liver weight was expressed proportionally to body weight, there were no significant differences between dietary groups. There were also no differences between dietary groups in the absolute or relative weight of epididymal fat, spleen and thymus.

2.4.4. DTH Response

In this study, ear swelling was measured 24 hours after injection with Influvac, and as such is a measure of DTH. Here, mice receiving tallow, sunflower or linseed oil diets had reduced ear swelling in response to Influvac compared to those receiving the salmon oil diet. The observations of a high response in the salmon oil group and the low response in the sunflower oil and beef tallow groups are in accordance with three of the hypotheses being tested in this study. In addition, DTH response was higher in salmon oil fed animals than linseed oil fed animals and there was no significant difference between linseed oil feeding, and sunflower oil or tallow feeding. This observation is not in agreement with the fourth hypothesis being tested in this study. The difference in DTH response between linseed oil and salmon oil may be due to the need to convert ALA to long-chain *n*-3 PUFAs especially DHA in order to see such a response. Erythrocyte DHA was higher in the salmon oil group than in all other groups, including the linseed oil group (erythrocyte EPA was similar between salmon oil and linseed oil groups), and it is possible that it is DHA which is responsible for the enhanced DTH response seen in the salmon oil group. However, using data from all animals irrespective of diet fed did not show a significant relationship between erythrocyte DHA and ear swelling ($r = 0.148$, $p = 0.376$). There was

however a significant inverse correlation between total erythrocyte *n-6* PUFAs and ear swelling ($r = -0.345$, $p = 0.036$), suggesting that it is exposure to *n-6* fatty acids that determines the strength of the DTH response. The salmon oil group showed the lowest total erythrocyte *n-6* fatty acids and this was lower than all other groups. Amongst the *n-6* fatty acids, the relationships between erythrocyte linoleic acid and arachidonic acid and ear swelling both tended towards significance ($r = -0.304$, $p = 0.067$ for linoleic acid and $r = -0.289$, $p = 0.083$ for arachidonic acid).

2.4.5. Plasma Immunoglobulin Concentrations

There was no effect of diet on plasma IgG2a, whilst IgG1 levels were below detection limits. IgG2a production is promoted by T_H1 cells, whilst T_H2 cells promote IgG1 production. The ability to measure IgG2a but not IgG1 is consistent with the idea that C57BL/6 mice are "T_H1 dominant". Previous research on the effect of dietary fatty acids on levels of different classes of immunoglobulins has produced conflicting results (see 1.4.5), and is further complicated by the fact that many papers examine the levels of total IgG rather than its subclasses. EPA supplementation (2.0 g/day for 7 weeks) has been shown to increase total IgG levels in plasma of healthy young men, when comparing baseline values to those taken at baseline [74]. There was no difference observed between EPA supplementation and those receiving GLA (2.0 g/d) or stearidonic acid in combination with GLA (1.0 g and 0.9 g/d respectively), although in these dietary groups, there was no effect observed between the start and end of supplementation. In the current study, plasma was not taken at the start of the trial, because of the size of the animals, which meant that insufficient sample would have been available. Therefore whether diet affected changes in IgG2a over time is not known from the current study. In addition, Miles *et al.* [74] looked only at total IgG levels. Research has not previously been carried out on the effect of dietary fatty acids on the level of IgG1 and IgG2a in plasma.

2.4.6. Cytokine Analysis

IFN-γ, IL-4 and IL-10 production by spleen cell cultures in response to Influvac was below detectable limits. IFN-γ is a signature cytokine of T_H1 cells, and IL-4 and IL-10 are signature cytokines of T_H2 cells. It cannot be determined whether dietary fatty acids affected the T_H1 / T_H2 cell balance in the current study based on these cytokines. It was not expected that these cytokines would be below detectable limits. Reasons for this may be that insufficient Influvac was used as a stimulant, that there were insufficient vaccine-

specific T-cells in the cultures to measure detectable cytokine responses, or that cells were not cultured for a long enough period for a response to be seen. The culture conditions used were chosen as they had previously been used in an Influvac vaccination model examining the effect of prebiotics on DTH, at the same department in the University of Utrecht where this experiment was carried out [158]. However, it may be that in this model, conditions may not be the same when examining the effect of fatty acids on DTH as for determining the effect of prebiotics.

2.4.7. Histological Analysis

The cortex/medulla ratio of thymuses was examined as previous research at the University of Utrecht had suggested that DHA may reduce medulla areas of the thymus (A. Hogenkamp, unpublished personal communication). This in turn could alter area available for T-cell maturation, or indicate an effect of maturation. However, there was no effect of fatty acids on cortex/medulla ratio of thymuses in this model.

2.4.8. Cell Analysis

There was no effect of dietary fatty acids on cell subsets in the thymus, spleen or bone marrow. This is in accordance with the findings of previous research papers reviewed in 1.4. Any effect on T_H1 / T_H2 balance that might have been expected would not be observed in this analysis, as T_H1 and T_H2 cells are indistinguishable by flow cytometry.

2.4.9. Limitations

One limitation of this study is that a maximum of eight animals per diet group were studied; a larger sample size may have revealed stronger findings. A second limitation is that fatty acid composition data for immune cells were not available and erythrocyte fatty acids were used to examine the relationship between fatty acid status and immune response. Using data for immune cells would be more appropriate for this purpose. The third limitation is that some of the immune responses assessed here were below the limit of detection and this meant that these were not useful. This is especially true for the cytokine profiles produced by cultured splenocytes. A longer culture period and/or a different concentration of the vaccine stimulant may have generated useable and useful data.

2.4.10. Conclusions

The study set out to test hypotheses of increased immune response to influenza vaccination with salmon and linseed oils and reduced response with sunflower oil and tallow. Data from measuring ear swelling, suggest that mice had an increased T_H1 response to vaccination following feeding with salmon oil, compared to linseed oil, sunflower oil or tallow, in accordance with some of the hypotheses being tested. However, analysis of plasma immunoglobulins showed no effect of diet on IgG2a levels, and plasma levels of IgG1 and spleen cell culture levels of IFN-γ, IL-4 and IL-10 were below detection limit, and therefore could not add weight to this conclusion. It may be that levels of IgG2a may have been affected by supplementation in comparison to baseline values, but this was not measured due to the size of animals at this point. Results from this chapter suggest that salmon oil may not be as detrimental to the immune response and to the response to immune challenges like infection as was suggested by Schwerbrock *et al.* [146], as ear swelling in response to Influvac was higher in the salmon oil fed mice than the linseed oil, sunflower oil or tallow fed mice. Although the difference in DTH response between linseed oil and salmon oil may be due to the need to convert ALA to DHA, which appears to be rate-limiting, the most important difference in fatty acid composition appears to be the lower *n-6* fatty acid status that occurs with salmon oil feeding.

Chapter 3. The Effect of Dietary Fatty Acids on a Mouse Model of Allergic Sensitisation

3.1. Introduction

The previous chapter showed that dietary fatty acids have the potential to affect T_H1 type immune responses. To develop this further, another area of the immune system of clinical importance to humans was then examined. In collaboration with the University of Utrecht, a study was completed in order to examine the effect of dietary fatty acids in a mouse model of allergic sensitisation. The author was involved in tissue and data collection on the day of euthanisation, whilst Dr. Astrid Hogenkamp and Dr. Naomi van Vlies at the University of Utrecht carried out feeding and experiments prior to this. The author analysed all data produced.

3.1.1. Atopy

Atopy is a term used to describe immediate hypersensitivity (Type I) reactions, where an inappropriate immune reaction is caused by the action of T_H2 cells. The pathway of immediate hypersensitivity is shown in Figure 3-1. Exposure to allergen causes antigen (i.e. allergen) activation of T_H2 cells, and IgE production by B-cells. IgE binds to FcεRI receptors on mast cells, and by doing so increases expression of these receptors [4]. Repeated exposure to the allergen activates mast cells, causing the release of mediators (vasoactive amines, lipid mediators and cytokines). The immediate phase reaction occurs minutes after allergen exposure, and is caused by release and actions of vasoactive amines and lipid mediators (leukotrienes, prostaglandins and thromboxanes). The late phase reaction occurs 2 – 4 hours after allergen exposure and is caused by the action of the cytokines released (IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-11, IL-13, GM-CDF, TNF, CCL2, CCL-5 and CCL11).

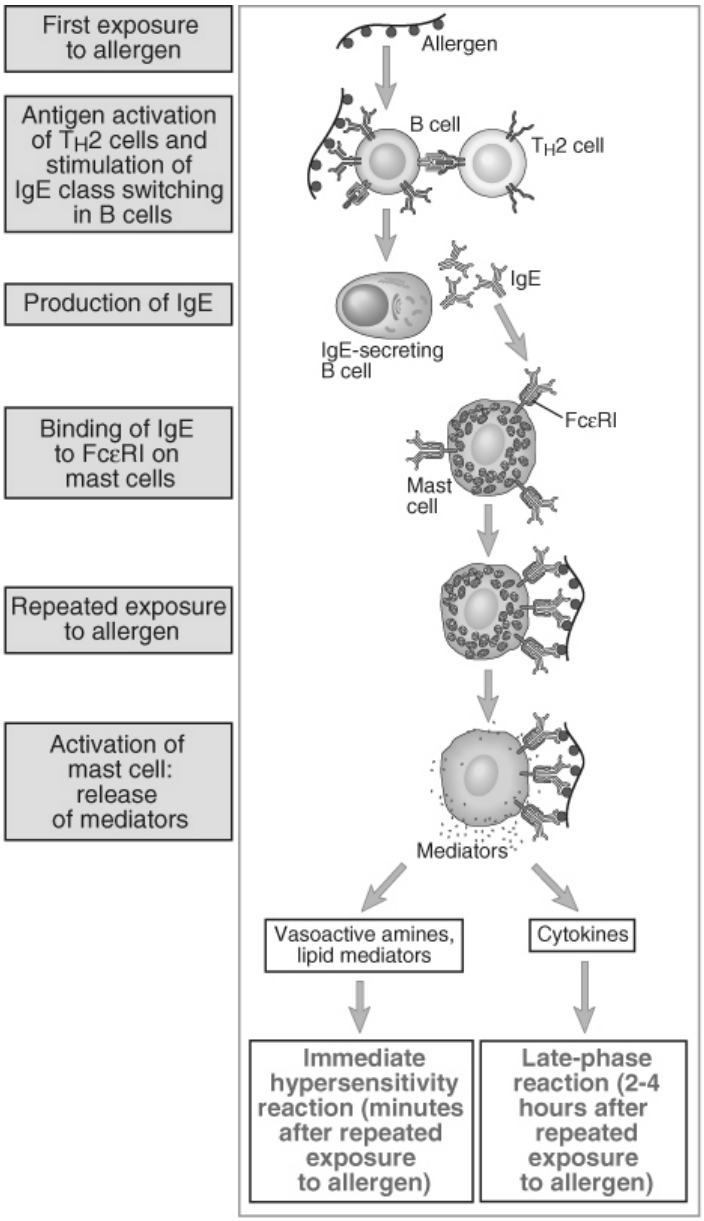

Figure 3-1: Pathway of atopy (immediate hypersensitivity). Exposure of allergen causes antigen activation of T_H2 cells, and IgE production by B-cells. IgE binds to FCεRI receptors on mast cells. Repeated exposure to the allergen activates mast cells, causing the release of mediators. Taken from Abbas and Lichtman [5].

Depending on the site of exposure to antigen, atopy can be manifested in different ways, and with either a localised or generalised effect, although all have essentially the same cause; exposure of a sensitised individual to an allergen results in mast cell activation via the pathway shown in Figure 3-1. For example, exposure of antigen to the respiratory tract can result in asthma or allergic rhinitis, whilst exposure to the eyes can cause conjunctivitis. Antigen exposure in the digestive tract can cause food allergies, presenting with vomiting and diarrhoea, whilst exposure to the skin can lead to atopic dermatitis or eczema.

3.1.2. Skin Prick Test / Acute Skin Response

Injection of antigen into the skin of a sensitised individual will result in a wheal and flare response (the immediate phase), followed by a late phase response resulting in eosinophil accumulation. This is often used as a method of determining sensitisation (i.e. an atopic response) to a particular allergen (skin prick testing or acute skin response); the subject is injected with small amounts of one or more antigen(s), and the resulting inflammation reaction recorded and used to assess whether the individual is allergic to the allergen(s) or, as in this study, the effect of treatments (e.g. diet) on reducing the allergic response. A visualisation of the immediate and late responses in a skin prick test in humans is shown in Figure 3-2.

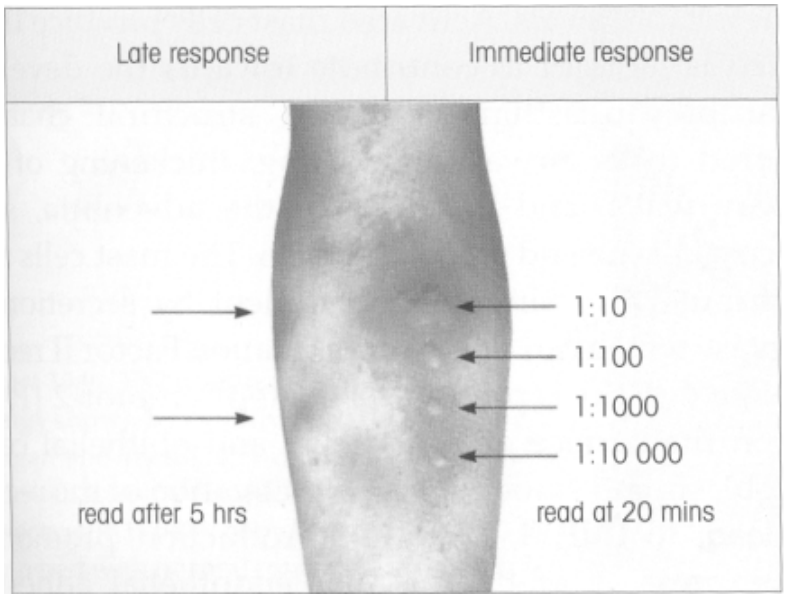

Figure 3-2: Skin prick test to grass pollen allergen in a patient with typical summer hayfever. Skin prick tests were carried out 5 hours (left) and 20 minutes (right) before the photograph was taken. The immediate response can be seen on the right, with different allergen dilutions exhibiting different degrees of wheal and flare reactions. The late response can be seen on the left. Taken from Delves *et al.* [4].

3.1.3. Asthma

Asthma is a type of immediate hypersensitivity reaction that is characterised by intermittent and reversible obstruction of the airway, chronic inflammation of the bronchus with eosinophilic infiltration, hypertrophy of bronchial smooth muscle, and hyper-reactivity to bronchoconstrictors [5]. There are three types of asthma; occupational asthma results from allergens in the workplace, extrinsic or allergic asthma is caused by atopy and a genetic predisposition to produce inappropriate levels of IgE; and intrinsic or idiopathic asthma, which is non-atopic, and the cause for which may not be known [4]. The study described in this chapter will focus on allergic asthma, using ovalbumin (egg protein) as the allergen.

3.1.4. Prevalence of Atopy

Epidemiological studies in the United Kingdom [159-162], Germany [163], Switzerland [164], USA [165, 166], Australia [167, 168] and New Zealand [169] have suggested an increasing prevalence of atopy. A more recent international study reports a generalised worldwide increase in asthma, allergic rhinitis and eczema prevalence [170]. The British Allergy Foundation estimates that one in four of the UK population will suffer from an allergy at some point in their lives [171] and Asthma UK reports that 5.4 million people in the UK are

currently receiving treatment for asthma [172], which amounts to 1 in 12 of the adults and 1 in 11 children in the UK population [173].

3.1.5. Dietary Fat and Atopy

Numerous suggestions have been made to explain the increase in prevalence of atopy, including environmental factors and genetic susceptibility [174]. One explanation for the general increase in prevalence of atopy has been diet, in particular a changed pattern of consumption of PUFAs. Consumption of *n-6* PUFAs (from margarine and vegetable oils) has increased over the period since 1960, whilst intake of *n-3* PUFAs and antioxidants (from fruit and vegetables) has decreased [41]. The mechanism behind the theory linking dietary PUFAs with increased atopy prevalence is that the major dietary *n-6* PUFA linoleic acid is converted to AA, which in turn is metabolised to PGE_2, TXA_2, and the 4-series leukotrienes (Figure 1-19). PGE_2 promotes differentiation of naïve T helper cells to the T_H2 phenotype which is pro-allergic (T_H2 cells promote IgE production via IL-4 while IL-5 activates eosinophils), and the 4-series leukotrienes, promote many of the manifestations of allergic disease, particularly at the level of the lung, such as bronchoconstriction and mucous production [41]. In contrast, as LA and ALA compete for the same enzyme, Δ6-desaturase (Figure 1-17), a higher intake of *n-3* PUFAs reduces AA abundance and its subsequent conversion to PGE_2 and 4-series leukotrienes. Instead, EPA is metabolised to PGE_3, TXA_3, and the 5-series leukotrienes, rather than the atopy-associated PGE_2 and 4-series leukotrienes (Figure 1-19). As a result the T_H1 / T_H2 balance becomes less polarised towards the T_H2 cell phenotype reducing the T and B cell drive towards atopy and, via reduced 4-series leukotrienes, reduced severity of allergic manifestations.

3.1.6. Mouse Model of Atopic Sensitisation

The mouse model of atopy is described by Hessel *et al.* [175]. Briefly, BALB/C mice (which have a genetic predisposition to a T_H2 predominant reaction [150]) receive an *intra-peritoneal* injection of ovalbumin to create an allergic sensitivity to the protein, and four weeks later can be challenged with aerosolised ovalbumin, in order to induce an asthma-like response. Following the aerosolised challenge lung function can be evaluated with a non-invasive oscillation technique, and independently of this several markers of the immune response can be determined, including mast cell degranulation, IgE levels in serum, and splenocyte cytokine profiles. By using this model, and feeding mice diets

containing different fatty acids, it is possible to examine their effect on the immune system using an accepted *in vivo* model of allergic sensitisation.

3.1.7. Aims of the study

This study aimed to determine the effect of diets differing in fatty acid composition, including different levels and different types of *n-3* and *n-6* fatty acids, on the immune response in a mouse model of allergy to the egg protein ovalbumin. Altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing them to become more or less susceptible to their allergy.

In order to achieve this aim, six diets were fed to mice from 3 to 4 weeks of age until 8 to 9 weeks of age. The diets were the same as those used in the previous chapter.

3.1.8. Hypotheses

This study set out to test the following hypotheses:

- Feeding the salmon oil diet will reduce the severity of allergic reactions
- Feeding the sunflower oil diet will increase the severity of allergic reactions
- Feeding the beef tallow diet will increase the severity of allergic reactions
- Feeding the linseed oil diet will decrease the severity of allergic reactions compared to the sunflower oil diet, as ALA can be converted to EPA, but will not be as inhibitory as the salmon oil diet, as fatty acid metabolism is a less effective way of producing EPA than direct provision in the diet.

3.2. Materials and Methods

3.2.1. Animals, Diets and Materials

3.2.1.1. Animals

3 to 4 week old male BALB/C mice were obtained from Charles River (Maastricht, The Netherlands). Animals were housed in groups of six in shoebox cages, and kept at 22°C ± 2°C, under a 12 hour light: 12 hour dark cycle in temperature and light-controlled room in the animal facility at the Pharmacology Department, University of Utrecht. Animals had *ad libitum* access to tap water and 150 g food was provided on alternate days. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

3.2.1.2. Diets

Diets were identical to those used in the previous chapter (see 2.2.1.2).

3.2.1.3. Materials

Ovalbumin (Grade V), methacholine, urethane and β-mercaptoethanol were obtained from Sigma (St. Louis, USA). Aluminium hydroxide was purchased from Pierce Chemical Company (Rockford, USA). Complete mini was obtained from Roche Applied Science (Burgess Hill, UK). Other chemicals used were from the same sources as used in the previous chapter.

3.2.2. Methods

An overview of the study design is provided in Figure 3-3. BALB/C mice (n = 48) were randomised to receive one of six diets (n = 8/diet) twelve days before sensitisation (at day -12). Sensitisation was carried out at days 0 and 7. Blood was collected by tail vein puncture on days -12, 14 and 22. Acute skin tests were carried out on day 21 ± 1. Mice were challenged on days 29, 32, and 35 ± 1. Airway responsiveness was measured on days 29 and 35 ± 1. Euthanisation occurred by *intra-peritoneal* injection of 1 ml 10 % urethane on day 36 ± 1.

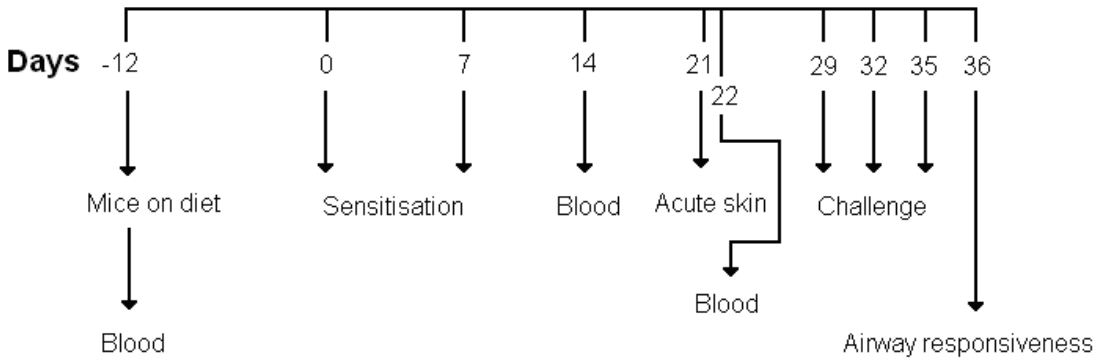

Figure 3-3: Schematic diagram of experiment examining the effect of dietary fatty acids on a mouse model of allergic asthma.

3.2.2.1. Sensitisation of Mice to Ovalbumin

Mice were sensitised with ovalbumin at days 0 and 7. Sensitisation was achieved through two *intra-peritoneal* injections of 10 µg ovalbumin adsorbed onto 22.5 mg aluminium hydroxide in 100 µl saline.

3.2.2.2. Acute Skin Test to Ovalbumin Challenge

An acute skin test was carried out at day 21. This used the same method as for the DTH response in the previous chapter, but 1 pg ovalbumin in 0.01 ml PBS was injected instead of Influvac, and ear swelling measured 1 hour, rather than 24 hours, after injection.

3.2.2.3. Determination of Airway Responsiveness to Ovalbumin

Mice were challenged with aerosolised ovalbumin at day 29 $\pm$ 1, 32 $\pm$ 1 and 35 $\pm$ 1, by exposure in a 5 L plexiglass exposure chamber. Aerosols were generated by nebulizing an ovalbumin solution (10 mg/mL in pyrogen-free saline) using a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 mm) driven by compressed air at a flow rate of 6 L/min. Airway responsiveness was measured on day 36 $\pm$ 1 by recording respiratory pressure curve changes in response to inhaled nebulised methacholine with barometric whole-body plethysmography (BUXCO, EMKA Technologies, Paris, France), and expressed with a measurement of airway obstruction, enhanced pause (P_{enh}), using the method of Hamelmann *et al.* [176]. Briefly, animals were placed in the BUXCO, and pressure difference between the animal chamber and a reference chamber noted. Baseline values of P_{enh} were obtained and averaged over three minutes. Animals were exposed to a series of aerosols, starting with a saline aerosol, and

followed by methacholine aerosols doubling in concentration, concentrations ranging from 1.56 to 50 mg/ml saline. Aerosols were generated using the Paris LC Star nebuliser previously described, for three minutes, and following each nebulisation, readings recorded for three minutes and averaged.

3.2.2.3.1. Enhanced Pause (Penh)

P_{enh} is a unit-less measurement of airway hyper-reactivity. It is devised from a calculation based on the magnitude of the peak expiratory pressure compared to the peak inspiratory pressure, as a factor of pause. Pause is calculated by dividing the difference between time taken to exhale and time taken to reduce to a set value of 36 % above the normal pressure of the plethysmograph, by the time taken to inhale. It is shown in Figure 3-4.

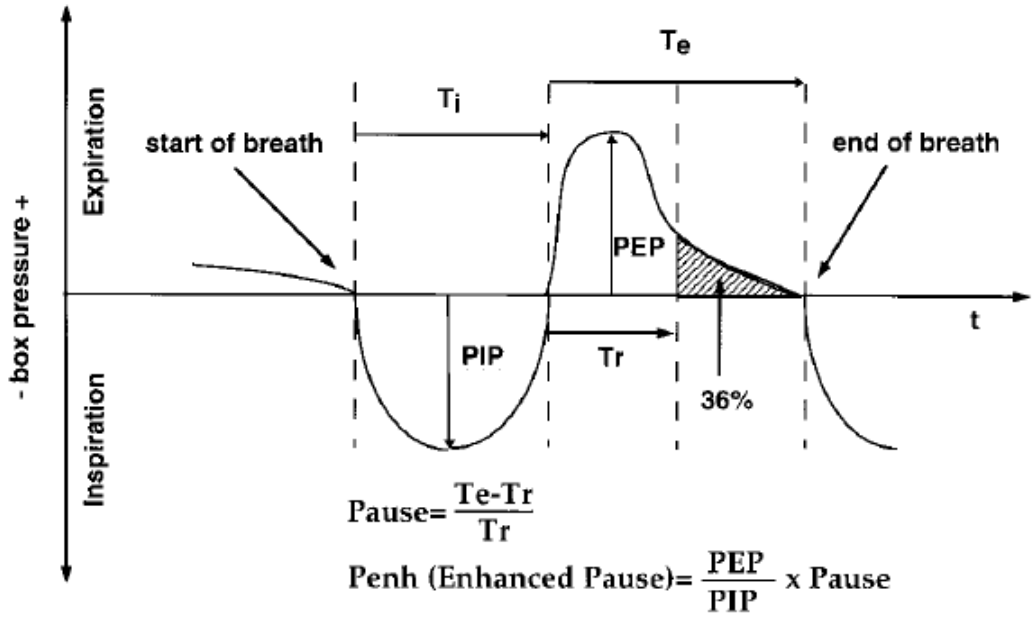

Figure 3-4: Calculation of P_{enh}. PEP, Peak expiratory pressure (ml / s); PIP, Peak inspiratory pressure (ml / s); T_e, Time taken for expiration (s); T_i, Time taken for inspiration (s); Tr, relaxation time (time taken for pressure to reduce to 36 % of the total box pressure during expiration. Taken from Hammelman *et al.* [176].

P_{enh} is not an accurate measure of resistance, but it is useful as an indicator of resistance. It was chosen for use in this research, as it allows respiratory measurements to be made on conscious, unrestrained animals, unlike other measurements of respiration [177].

3.2.2.4. Fatty Acid Analysis of Erythrocytes

At euthanisation, blood was collected, and erythrocytes analysed for fatty acids using the method previously described (2.2.2.3).

3.2.2.5. Analysis of Plasma Immunoglobulin Concentrations

Plasma, collected at euthanisation, was analysed for IgG1, IgG2 and IgE using sandwich ELISAs, following the manufacturer's instructions.

3.2.2.6. Analysis of Bronchoalveolar Lavage Fluid

Following blood collection, lungs were immediately washed with 1 ml PBS containing 5 % BSA and 1 tablet complete mini. This was then followed with three 1 ml washes of pyrogen free saline, which were pooled and stored on ice. These samples of bronchoalveolar lavage fluid (BALF) were used for IL-5 (see 3.2.2.7) and cytospin analysis (see 3.2.2.8).

3.2.2.7. Determination of IL-5 Content of BALF

Supernatants of the first millilitre of BALF were collected following centrifugation at 1500 rpm (500 g) for 5 min. These were analysed for IL-5 by sandwich ELISA using antibody pairs and standards, according to the manufacturer's instructions. The lower detection limit of the ELISA was 2 pg/mL.

3.2.2.8. Cytospin Analysis of BALF

The cell pellet of the first ml was added to the second to fourth ml of BALF collected. This cell suspension was centrifuged at 1500 rpm (500 g) for 5 min at 4 °C, and resuspended in 0.15 ml cold PBS. The number of cells present was determined using a Bürker-Türk counting-chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany). Cytospin preparations were made by centrifugation at 1500 rpm for 10 min. Cells were fixed and stained with Diff-Quick. In each Cytospin, at least 200 cells were counted and classified into mononuclear cells (monocytes, macrophages and lymphocytes) and eosinophils and neutrophils using standard morphology and staining characteristics.

3.2.2.9. Analysis of Immune Tissue, Fat and Liver

Spleen, thymus, epididymal fat and liver were removed and processed using the method described in the previous chapter (2.2.2.5).

3.2.2.10. Histological Analysis of the Thymus

Histological analysis of thymus was carried out using the method described in the previous chapter (2.2.2.6).

3.2.2.11. Determination of Cytokine Production by Splenocytes

Cultures of spleen cells were prepared as for the previous chapter and analyzed for IFN-γ, IL-4 and IL-10 production (2.3.7). However, cells were cultured with 0.1 μg/ml ovalbumin or anti-CD3, rather than Influvac.

3.2.2.12. Determination of Cell Subsets in Lymphoid Tissues using Flow Cytometry

Single cell suspensions of spleen, thymus and bone marrow were prepared and analysed for cell subsets following the methods of the previous chapter (2.2.2.8).

3.2.2.13. Statistical Analysis

Results were analysed using SPSS version 15.0. Parameters were analysed using Levene's test of homogeneity to ensure groups had equal variances, in which case they were compared using one-way analysis of variance (ANOVA), followed by *post-hoc* testing (Tukey). If Levene's test showed unequal variances to be present, groups were compared using the Kruskal-Wallis test. Where this was used, it is reported in the results section. If the Kruskal-Wallis test indicated a significant effect, Mann-Whitney U independent group comparisons tests were then carried out to compare each combination of groups.

3.3. Results

3.3.1. Body weights of Mice Fed the Different Diets

Mice were fed the diets for 48 days ± 1. Body weights of mice fed the different experimental diets are shown in Figure 3-5.

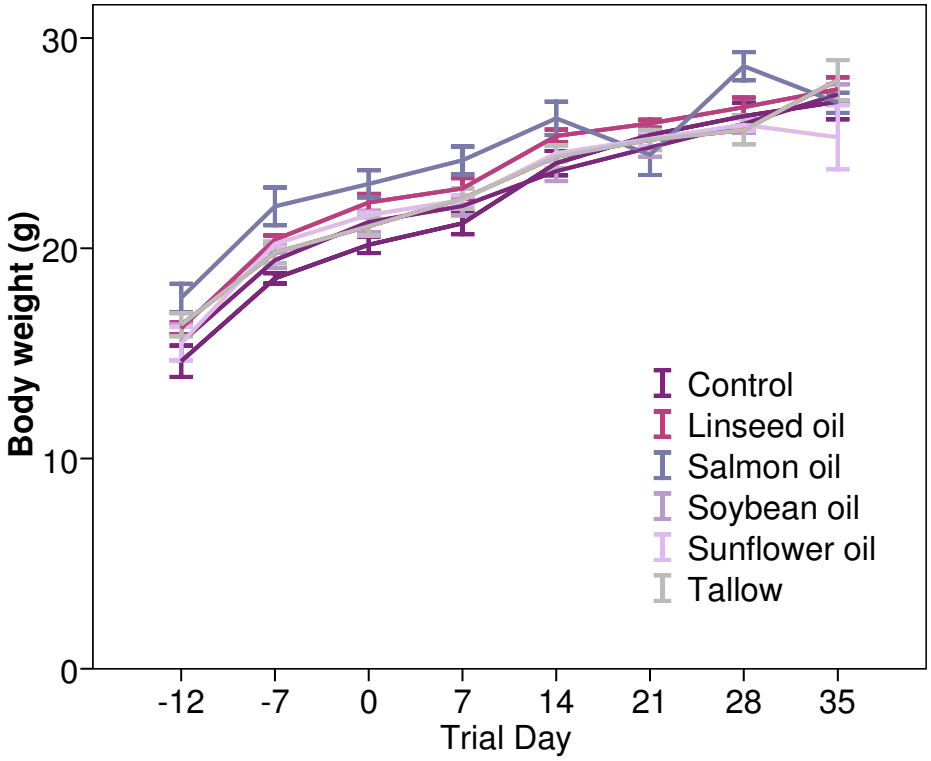

Figure 3-5: Body weights of mice fed the different diets. Data are mean ± SEM.

At day -14 and 0, mice in the salmon oil group were heavier than those in the control group. At day 0, mice in the salmon oil group were also heavier than those in the tallow group. At day -7 and 28, the salmon oil fed mice were heavier than those in the control, soybean oil and tallow groups. At day 7, the salmon oil fed mice were heavier than those in the soybean oil group. At all other time points, including the day of tissue collection (day 36; data not shown), there was no difference in body weight between dietary groups.

3.3.2. Tissue weights of Mice Fed the Different Diets

Tissue weights of mice fed the different diets are shown in Table 3-1.

Table 3-1: Tissue weights of mice fed the different diets. Fat refers to epididymal fat. Data are mean ± SEM. Values with different letters are significantly different from one another ($p < 0.05$). BW, Body weight.

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
Spleen (g)	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.21 ± 0.01
Spleen (% BW)	0.70 ± 0.04	0.73 ± 0.03	0.71 ± 0.04	0.69 ± 0.03	0.69 ± 0.02	0.74 ± 0.02
Thymus (g)	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
Thymus (% BW)	0.23 ± 0.01	0.23 ± 0.02	0.23 ± 0.02	0.24 ± 0.01	0.22 ± 0.01	0.23 ± 0.02
Liver (g)	1.45 ± 0.07	1.62 ± 0.05	1.55 ± 0.04	1.56 ± 0.07	1.53 ± 0.12	1.55 ± 0.09
Liver (% BW)	5.36 ± 0.10	5.89 ± 0.25	5.89 ± 0.17	5.72 ± 0.20	6.04 ± 0.24	5.52 ± 0.20
Fat (g)	0.38 ± 0.04^{ab}	0.38 ± 0.03^{ab}	0.37 ± 0.03^{ab}	0.40 ± 0.03^{ab}	0.29 ± 0.05^{a}	0.47 ± 0.03^{b}
Fat (% BW)	1.40 ± 0.15	1.36 ± 0.10	1.34 ± 0.11	1.47 ± 0.09	1.13 ± 0.18	1.66 ± 0.10

Epididymal fat (g) was heavier in animals in the tallow group compared to those in the sunflower oil group. However, when epididymal fat was analysed as a percentage of body weight there were no significant differences among the diet groups. There was no effect of diet on the weight of any of the other tissues examined.

3.3.3. Fatty Acid Content of Erythrocytes from Mice Fed the Different Diets

Erythrocytes were analysed for fatty acid composition. Data for all fatty acids is shown in Appendix Two, and data for fatty acids of interest is shown in Figure 3-6.

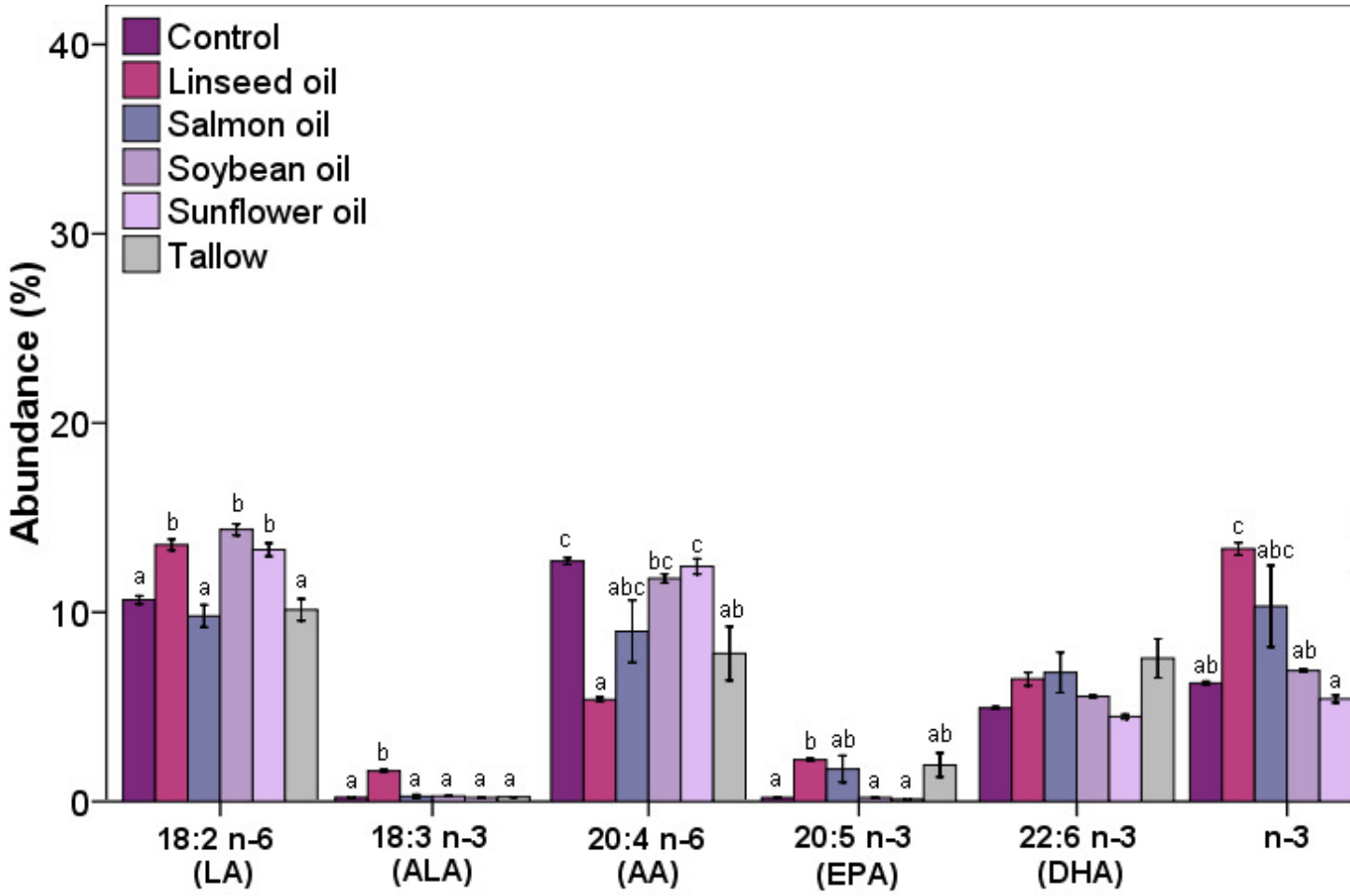

Figure 3-6: Individual and total *n-3* and *n-6* PUFA contents of erythrocytes from mice fed the different diets. Data are mean ± SE denote differences between dietary groups.

The linseed oil, soybean oil and sunflower oil groups had highest LA content of erythrocytes. The linseed oil group erythrocytes showed the highest ALA content. AA content was higher in erythrocytes from the sunflower oil and control groups compared to the tallow and linseed oil. AA content was higher in erythrocytes from the soybean oil group than the linseed oil group. EPA content was lower in the sunflower oil, soybean oil and control groups than the linseed oil group. There was no difference in DHA content of erythrocytes between different dietary groups. Linseed oil and tallow groups showed a higher *n*-3 content of erythrocytes than control, soybean oil and sunflower oil groups. Erythrocytes from the linseed oil, salmon oil and tallow groups had lower *n-6* content than erythrocytes from the soybean oil and sunflower oil groups. Erythrocytes from the control, soybean oil and sunflower oil diets had a similar ratio of *n-3* to *n-6* PUFAs; linseed oil, salmon oil and tallow group erythrocytes also had a similar *n-3* to *n-6* ratio to one another.

3.3.4. Airway Responsiveness in Mice Fed the Different Diets

The effect of the diets on airway responsiveness is shown in Figure 3-7. There was no difference between dietary groups at any dose of methacholine used.

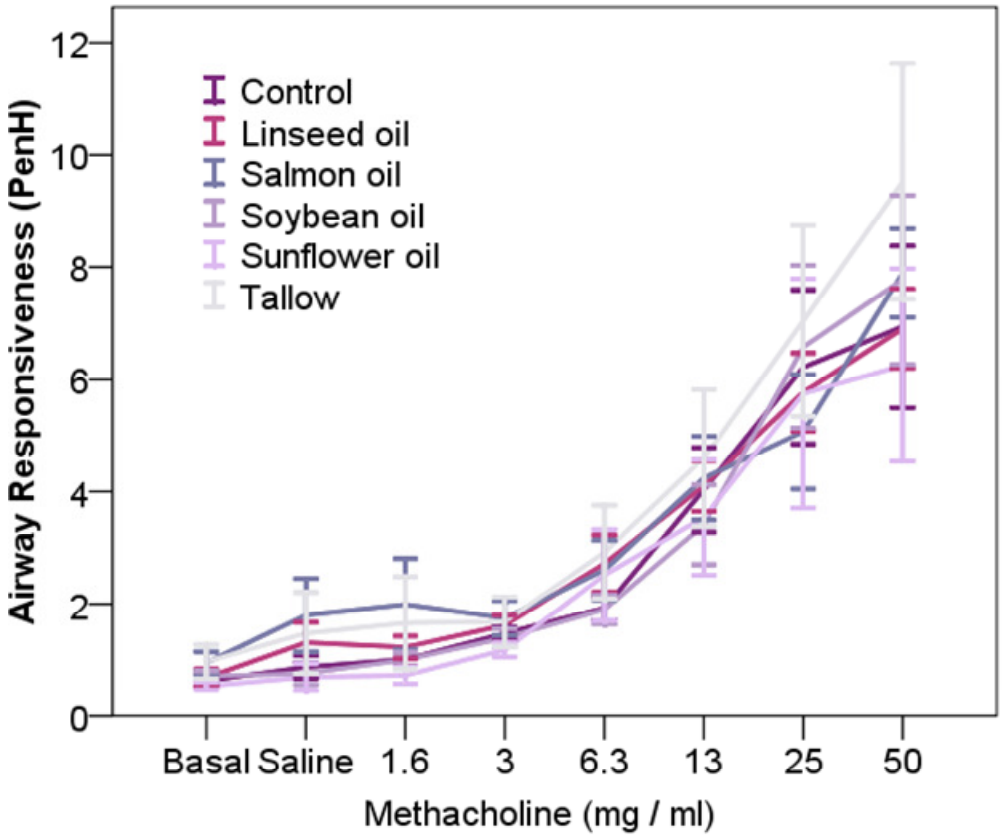

Figure 3-7: Airway responsiveness to increasing doses of methacholine in mice fed the different diets. Airway responsiveness was measured at day 36. Data are mean ± SEM.

3.3.5. Acute Skin Test in Mice Fed the Different Diets

The effect of the experimental diets on ear swelling in response to ovalbumin is shown in Figure 3-8.

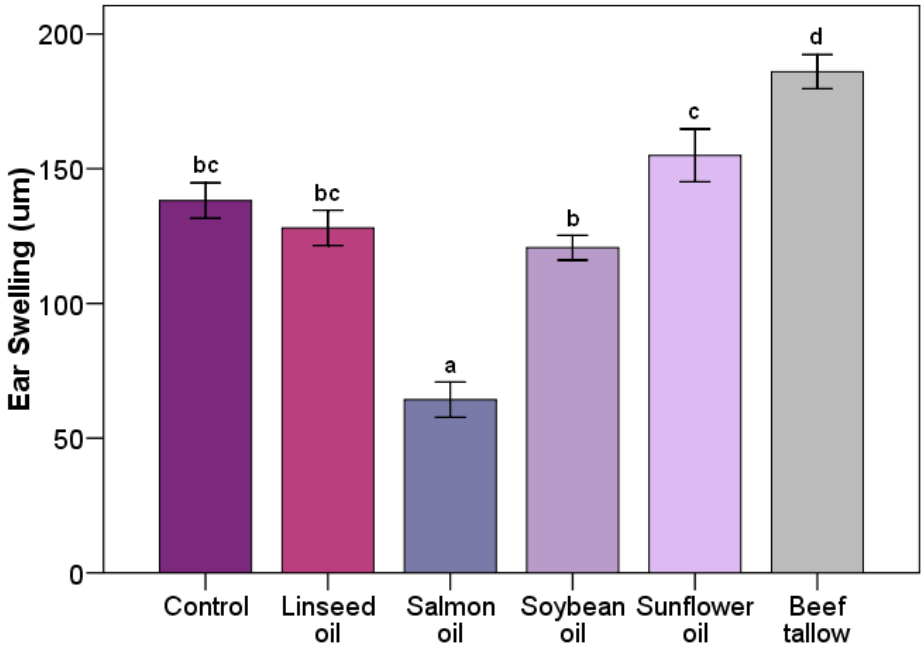

Figure 3-8: Ear swelling in response to ovalbumin in ovalbumin-sensitive mice fed the different diets. Data are mean ± SEM. Bars with different letters are significantly different from one another ($p < 0.05$).

Ear swelling was lowest in animals receiving the salmon oil diet; in this group swelling was significantly less than seen in each of the other groups. The reduction in response was about 50%. Animals receiving the soybean oil diet showed reduced ear swelling compared to those receiving the sunflower oil diet. Animals receiving the beef tallow diet showed the greatest ear swelling and this was significantly greater than seen in all other groups.

3.3.6. Plasma Immunoglobulin Concentrations in Mice Fed the Different Diets

Antibody concentrations in plasma are shown in Figure 3-9 (IgE), Figure 3-10 (IgG1) and Figure 3-11 (IgG2). IgE concentration in plasma was lower in mice receiving the soybean oil diet compared to the tallow diet. There was no effect of diet on IgG1 or IgG2 concentrations, although the latter was lowest in the sunflower oil group.

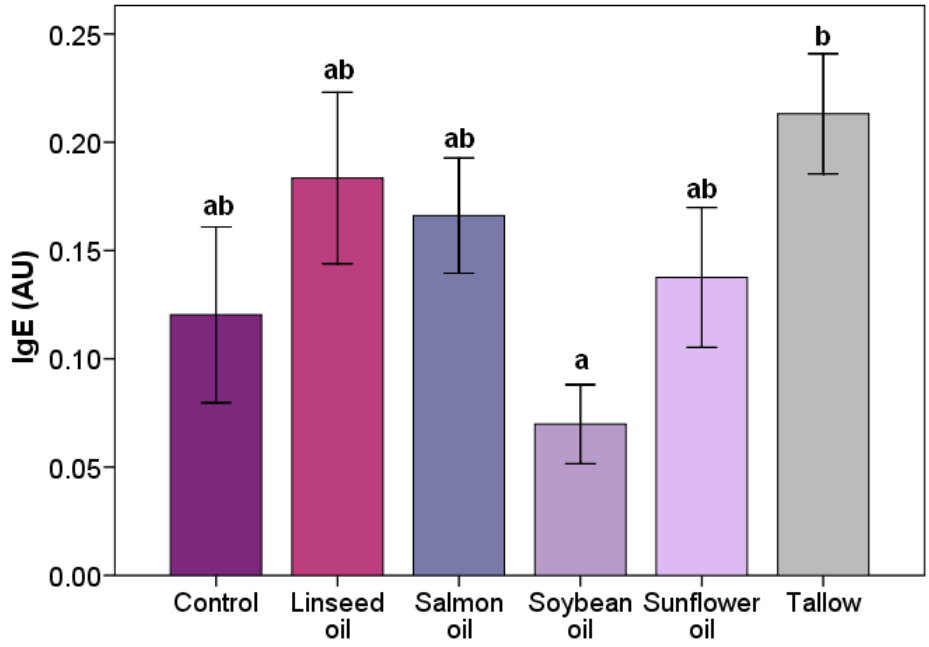

Figure 3-9: Plasma IgE concentration in mice fed the different diets. Data are mean ± SEM. Bars with different letters are significantly different from one another ($p < 0.05$).

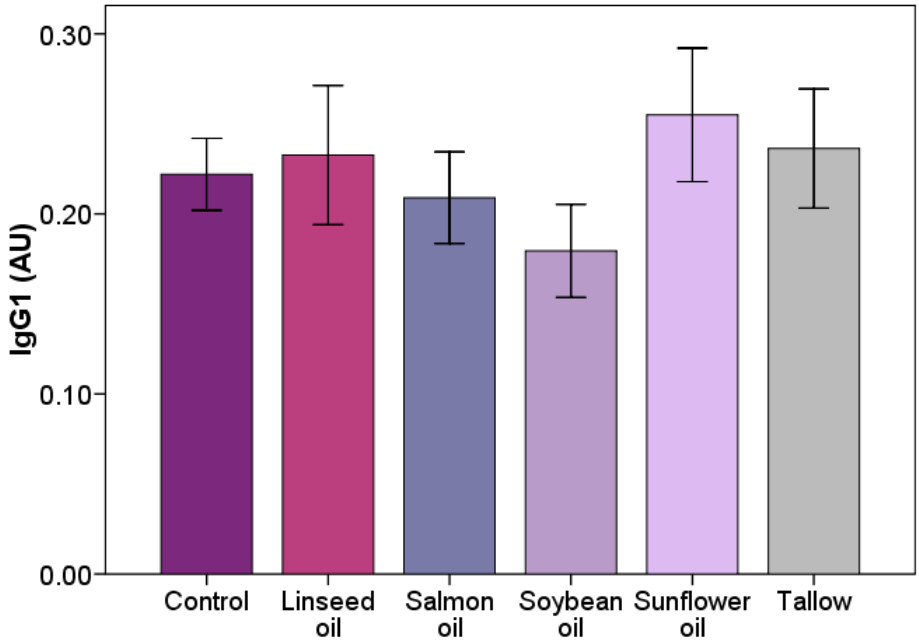

Figure 3-10: Plasma IgG1 concentration in mice fed the different diets. Data are mean ± SEM.

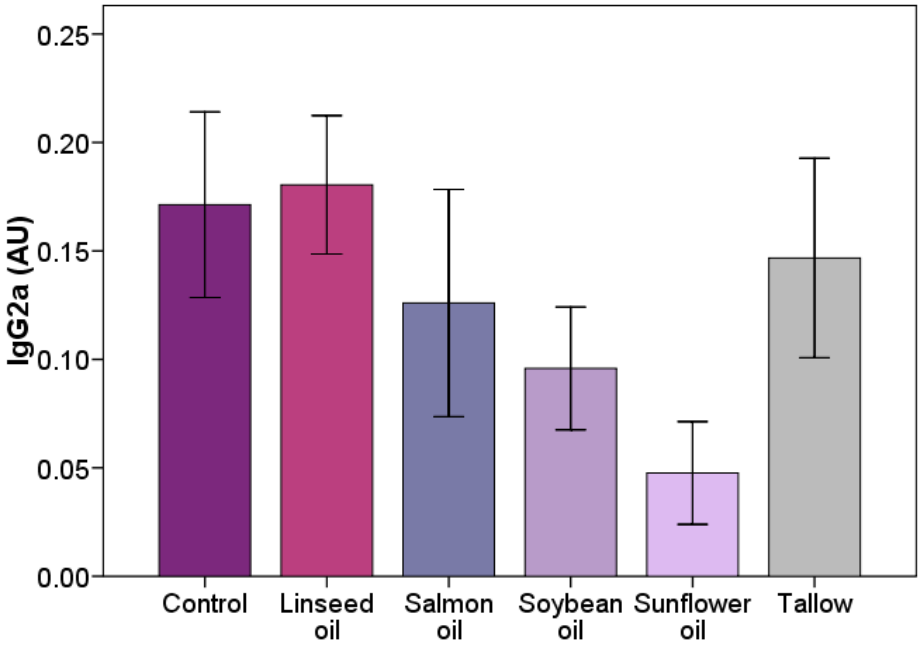

Figure 3-11: Plasma IgG2a concentration in mice fed the different diets. Data are mean ± SEM.

3.3.7. Cytokine Production by Cultured Splenocytes from Mice Fed the Different Diets

CD3- and ovalbumin -stimulated production of IL-4 and IL-10 by cultured spleen cells is shown in Figure 3-12 to Figure 3-15: AntiCD3-stimulated IL-10 production by spleen cells from mice fed the different diets. Data are mean + SEM.

There was no effect of diet on IFN-γ (data not shown), IL-10 production or ovalbumin-stimulated IL-4 production. Anti CD3-stimulated IL-4 production was lower in the control and sunflower oil groups than in the linseed oil and tallow groups.

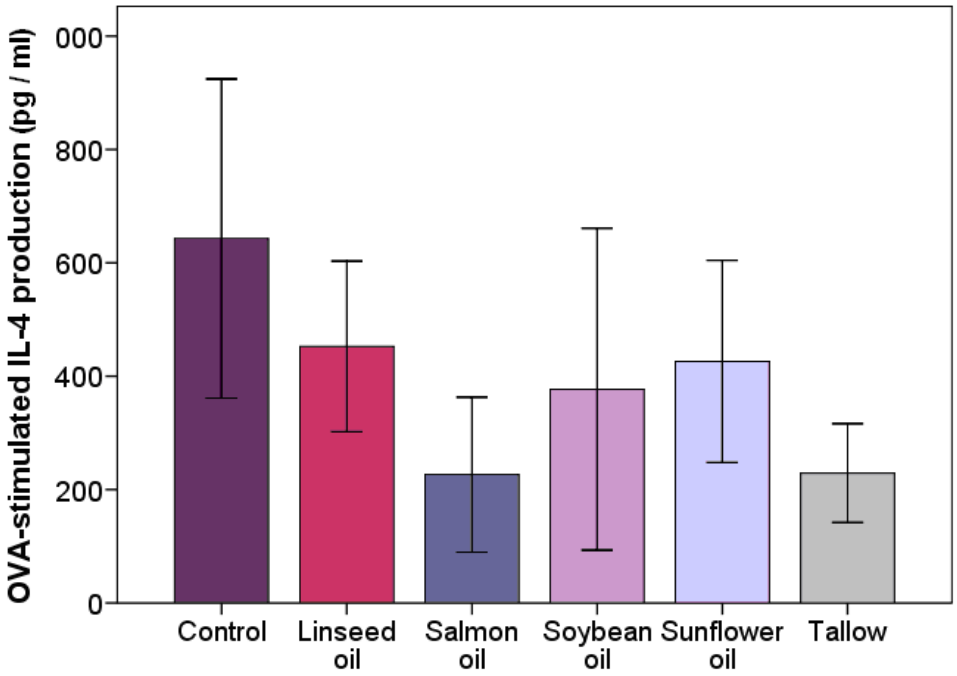

Figure 3-12: Ovalbumin-stimulated IL-4 production by spleen cells from mice fed the different diets. Data are mean + SEM.

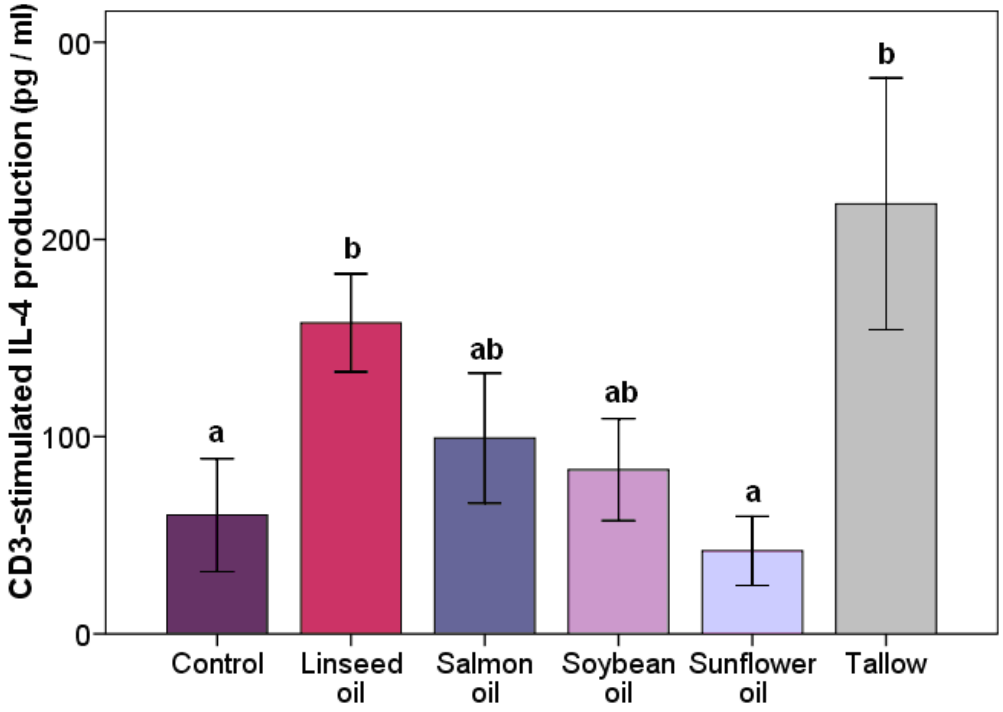

Figure 3-13: AntiCD3-stimulated IL-4 production by spleen cells from mice fed the different diets. Data are mean ± SEM. Bars with different letters denote significant differences between groups ($p < 0.05$). Data were analysed using non-parametric testing.

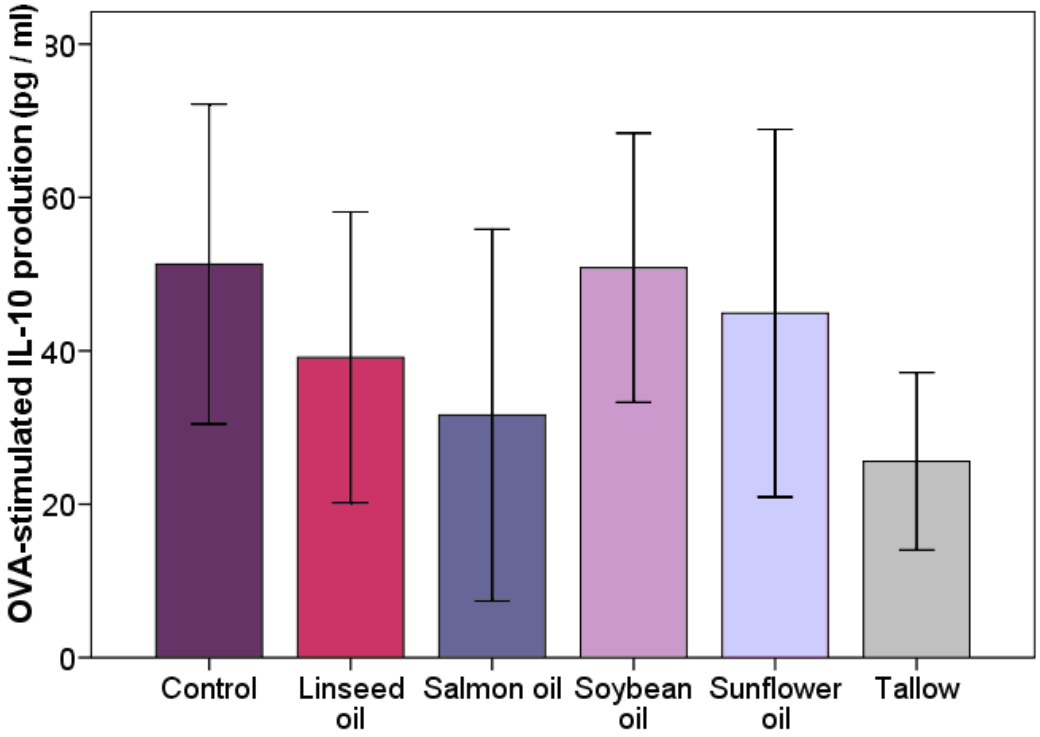

Figure 3-14: Ovalbumin-stimulated IL-10 production by spleen cells from mice fed the different diets. Data are mean + SEM.

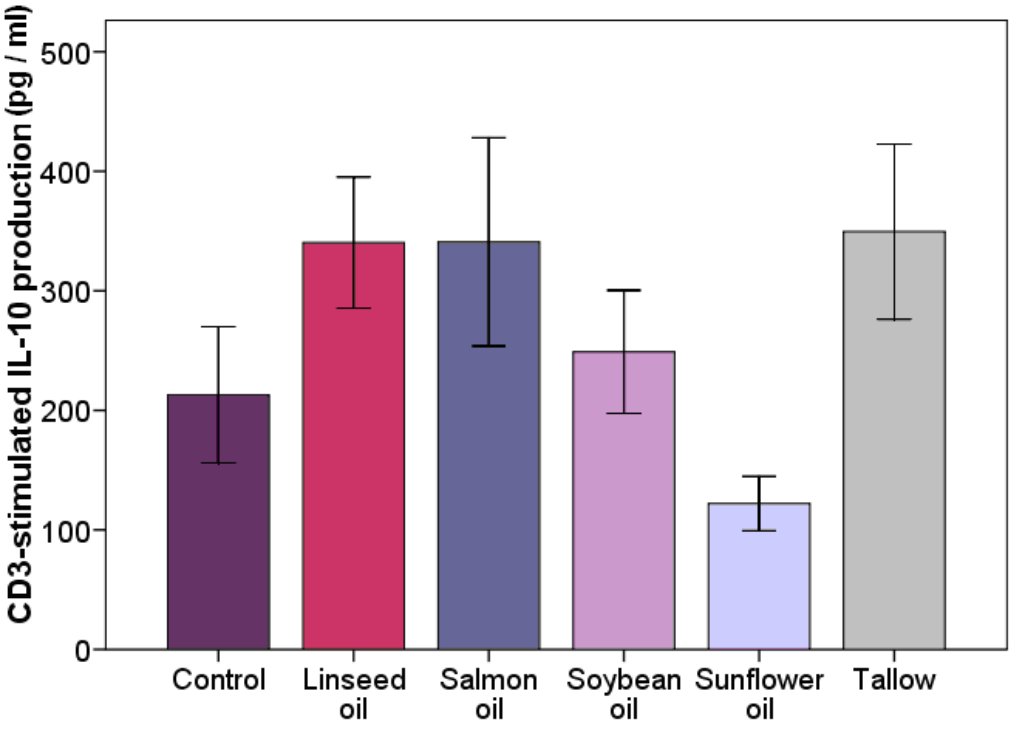

Figure 3-15: AntiCD3-stimulated IL-10 production by spleen cells from mice fed the different diets. Data are mean + SEM.

3.3.8. IL-5 Content of BALF from Mice Fed Different Diets

IL-5 content of BALF is shown in Figure 3-16. There was no significant difference between dietary groups.

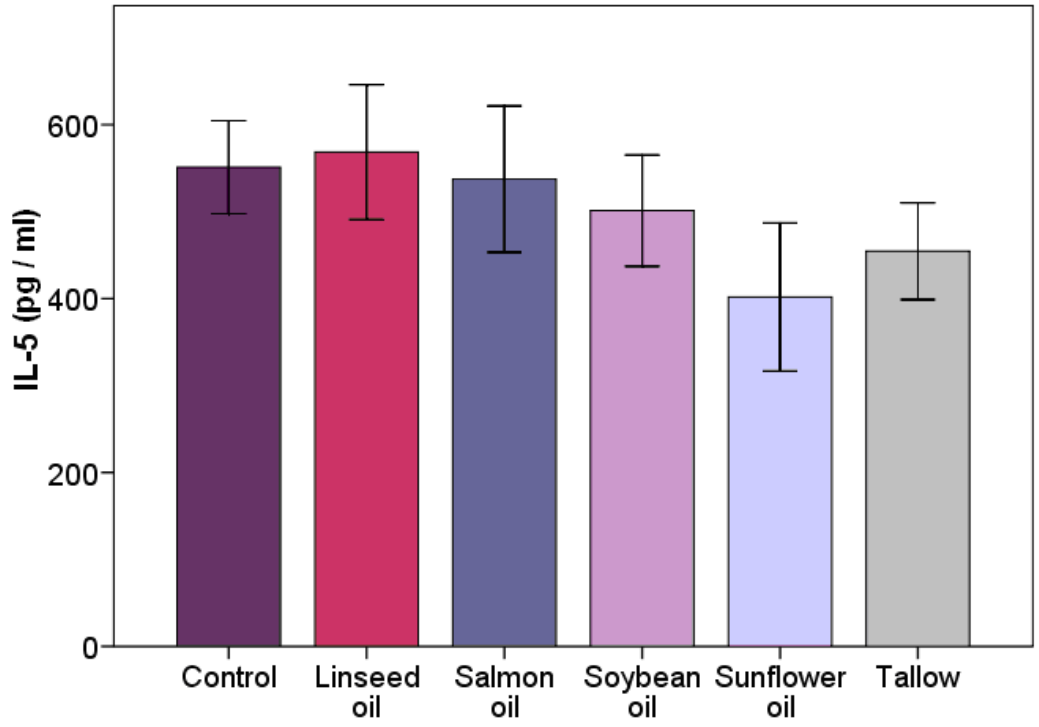

Figure 3-16: IL-5 concentration in BALF from mice fed the different diets. Data are mean + SEM.

3.3.9. Cytospin Analysis of BALF from Mice Fed the Different Diets

Results from the cytospin analysis of the bronchoalveolar lavage fluid (BALF) are shown in Figure 3-17. There was no effect of diet on any of the parameters measured. Cells described as “Other” in Figure 3-17 comprise mainly epithelial cells and basophils. Mononuclear cells include monocytes, macrophages and lymphocytes.

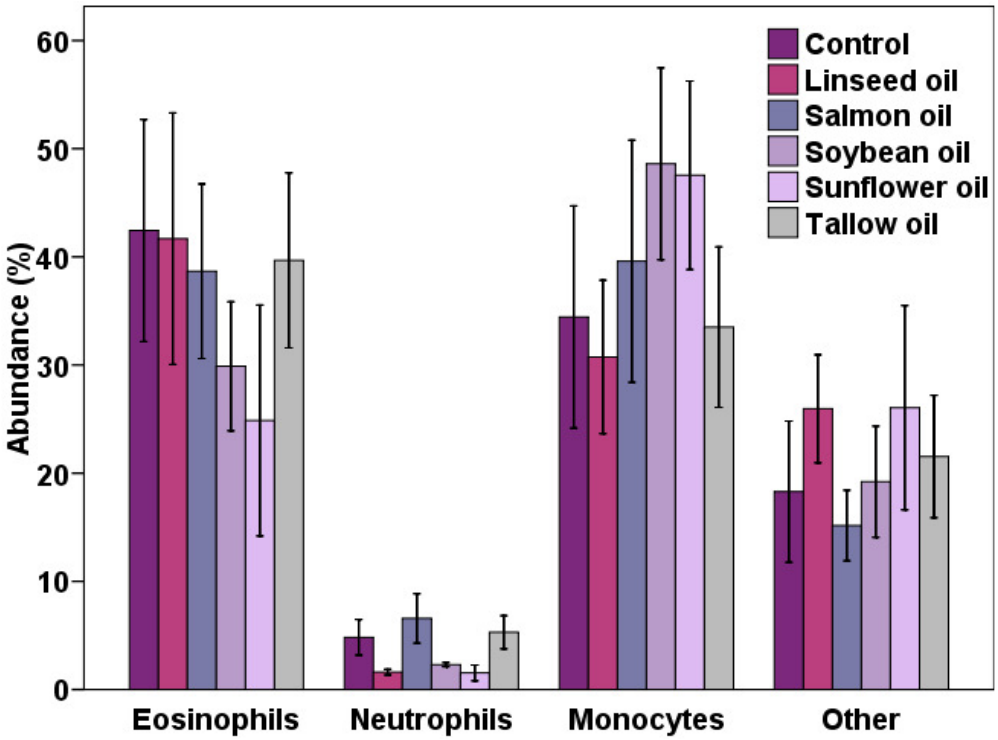

Figure 3-17: Cytospin analysis of bronchoalveolar lavage fluid from mice fed the different diets. Data are mean ± SEM.

3.3.10. Histological Analysis of Thymuses from Mice Fed the Different Diets

The cortex/medulla ratio of the thymuses from mice fed the different diets is shown in Figure 3-18. There were no significant differences between dietary groups.

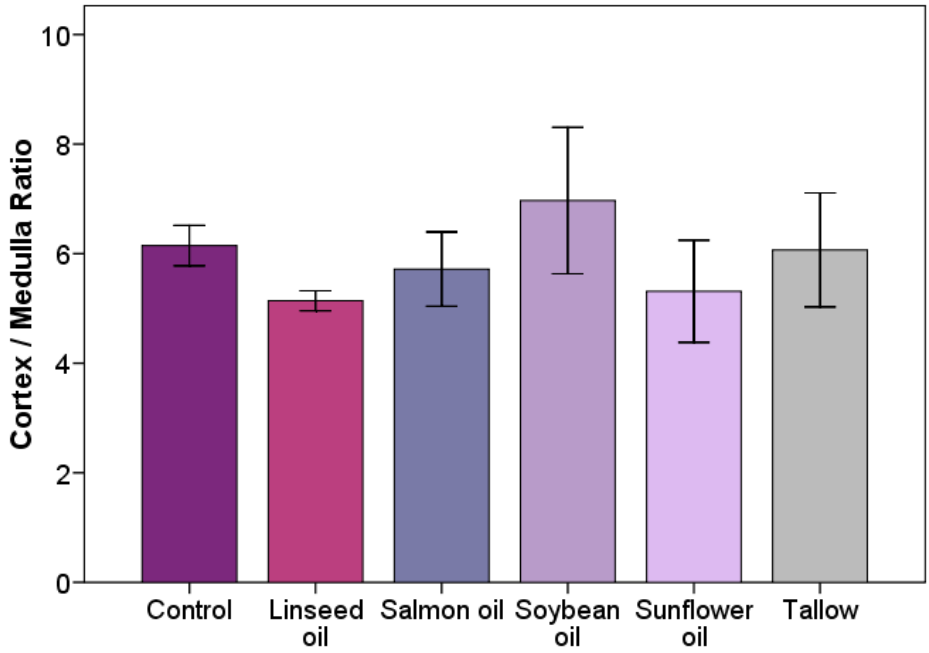

Figure 3-18: Cortex / Medulla ratio of thymuses from mice fed the different diets. Data are mean ± SEM.

3.3.11. Cell Subsets in the Thymus, Spleen and Bone Marrow from Mice Fed the Different Diets

The effect of the diets on the presence of different types of cell in spleen, thymus and bone marrow of mice is shown in Table 3-2 to Table 3-4. There was no effect of diet on the proportion of any of the cell types investigated in spleen, thymus or bone marrow.

Table 3-2: Cell subsets within the thymus of mice fed the different diets. Data are mean + SEM.

Marker	Cells Identified	Control	Soybean oil	Sunflower oil	Salmon oil	Linsee
CD3+	T-cells	95.38 ± 0.65	94.91 ± 0.83	95.09 ± 0.80	95.68 ± 0.37	96.18 ±
CD3+ that are also CD4+	T_H cells	92.42 ± 0.99	92.76 ± 0.74	91.28 ± 1.34	93.87 ± 0.59	90.76 ±
CD3+ that are also CD8+	CTLs	89.75 ± 1.07	88.60 ± 0.76	87.63 ± 1.29	89.94 ± 0.49	89.00 ±
CD3+CD4+ that are also CD62L+	L-selectin expressing T_H cells	82.19 ± 3.90	84.89 ± 4.21	87.67 ± 3.76	89.83 ± 4.12	88.12 ±
CD4+CD25+	T_{REG} cells	2.45 ± 0.82	1.67 ± 0.25	0.76 ± 0.14	1.05 ± 0.38	0.97 ±
CD4+CD25+ that are also FoxP3+	T_{REG} cells	0.64 ± 0.37	1.73 ± 0.97	5.08 ± 2.19	4.74 ± 3.23	3.12 ±

Table 3-3: Cell subsets within the spleen of mice fed the different diets. Data are mean + SEM.

Marker	Cells Identified	Control	Soybean oil	Sunflower oil	Salmon oil	Linsee
F4/80	Macrophages	5.73 ± 1.00	5.24 ± 0.56	8.82 ± 3.56	6.76 ± 1.04	7.26 ±
CD3+	T-cells	31.25 ± 1.35	27.72 ± 1.23	27.64 ± 1.92	28.90 ± 1.01	26.29 ±
CD3+ that are also CD4+	T_H cells	62.14 ± 4.75	61.14 ± 5.44	56.98 ± 5.63	54.24 ± 3.92	54.58 ±
CD3+ that are also CD8+	CTLs	2.00 ± 0.26	2.12 ± 0.32	2.17 ± 0.22	2.45 ± 0.32	1.56 ±
CD3+CD4+ that are also CD62L+	L-selectin expressing T_H cells	51.31 ± 2.68	49.78 ± 2.37	49.89 ± 2.07	51.26 ± 2.91	49.07 ±
CD3+CD4+ that are also CD69+	Activated T_H cells	43.80 ± 1.69	43.09 ± 2.30	46.59 ± 2.25	44.77 ± 2.33	48.46 ±
CD19	B-cells	23.45 ± 5.48	17.18 ± 1.82	21.97 ± 4.75	16.82 ± 1.79	19.25 ±
CD4+CD25+	T_{REG} cells	5.30 ± 0.33	4.70 ± 0.53	4.84 ± 0.76	4.26 ± 0.68	4.07 ±
CD4+CD25+ that are also FoxP3+	T_{REG} cells	7.97 ± 2.08	7.09 ± 1.55	6.99 ± 1.94	5.41 ± 1.59	8.26 ±
CD94+	NK cells	36.85 ± 1.82	28.09 ± 3.60	28.51 ± 4.90	24.40 ± 5.72	27.35 ±

Table 3-4: B cells within the bone marrow of mice fed the different diets. Data are mean + SEM.

Marker	Cells Identified	Control	Soybean oil	Sunflower oil	Salmon oil	Linseed oil	Beef tall
CD19+	B-cells	27.75 ± 2.26	26.19 ± 3.71	26.29 ± 4.80	18.99 ± 5.68	26.88 ± 5.02	30.41 ± 2

3.4. Discussion

3.4.1. Summary of findings

This study aimed to determine the effect of diets differing in fatty acid composition, including different types of *n-3* and *n-6* fatty acids, on the immune response in a mouse model of allergy to the egg protein ovalbumin. The study set out to test hypotheses of reduced severity with salmon and linseed oils and increased severity with sunflower oil and beef tallow. Using ear swelling in response to ovalbumin, to which the mice had been sensitised, as the outcome, salmon oil decreased the response and sunflower oil and tallow increased the response supporting the hypotheses about these fat sources. Linseed oil was without effect. In addition, tallow increased IgE production in plasma compared to soybean oil. Despite the effects of dietary fatty acids on ear swelling and IgE production there were no effects with regard to lung function or to immune markers measured in BALF, plasma, thymocytes, bone marrow or splenocytes.

There was an increase in body weight over the 48 days of the trial; at various points throughout the trial there were differences in body weight between dietary groups, but there was no difference between groups at the start or end of the trial. There was no effect of diet on spleen, thymus or liver weight. Epididymal fat was higher in the tallow group than the sunflower oil group, when measured as an absolute figure, but there was no difference seen when data was analysed as a percentage of body weight. Ear swelling in response to ovalbumin was lower in the salmon oil group than the soybean oil group, which in turn was lower than that of the sunflower oil group. Animals receiving the tallow diet had the greatest ear swelling in response to ovalbumin. There was no effect of diet on airway responsiveness. The fatty acid content of erythrocytes varied between dietary groups and generally reflected the fatty acid content of the diet. There was no effect of diet on IgG1 or IgG2 concentration in plasma, IL-5 content of BALF or on cytospin analysis of BALF. IgE content of plasma was lower in mice receiving the soybean oil diet compared to those receiving the tallow diet. The cortex / medulla ratio of mice thymuses was not affected by diet. There was no effect of diet on ovalbumin or CD3 stimulated IFN-γ, IL-10 production or ova-stimulated IL-4 production of splenocytes. CD3-stimulated IL-4 production by splenocytes was lower in the control and sunflower oil group than in the linseed oil and tallow groups. Diet did not affect cell subsets in the spleen, thymus or bone marrow.

3.4.2. Tissue and Body Weights

Body weights of mice increased over the period they received the experimental diets (48 days ± 1), in agreement with the findings of Poiley [157] and the previous chapter. Differences in body weights between dietary groups seen at various time points through the trial may have been caused by factors such as stress or palatability affecting appetite. However, since food intake was not recorded, and animals were housed in groups of 8, it is impossible to ascertain whether this occurred. Body weights did not differ between dietary groups at the start or the end of the trial, suggesting that this factor is unlikely to have confounded results. Differences in body and tissue weights between the current and previously described trial may be due to differences in duration of the trial, nature and timing of immune challenge(s), and strain of mice.

Yaqoob *et al.* [178] found epididymal fat pad weight to be smaller in safflower oil-fed rats than hydrogenated coconut oil fed rats. In addition, the same study also showed a menhaden oil-fed rats to have a smaller epididymal fat pads than safflower oil-fed rats; the current study found no difference between epididymal fat pads in the salmon oil group and the sunflower oil group. This may be due to the higher fat content of the Yaqoob study (20 %) or the fact that diet was fed to rats for ten weeks, whereas the current study fed mice for less than seven weeks, or perhaps even the differences in fatty acids between the safflower / menhaden oils and sunflower / salmon oils. Bowen and Clandinin [179] found no difference in effect of a high or low ALA diet in the first two weeks of life on epididymal fat pad weight in rats. Demar *et al.* [180] found no effect of inclusion of DHA in the diet on spleen, liver, white adipose or visceral adipose weight in young rats.

However, in this study, when data was analysed as a percentage of body weight, there was no longer a difference seen between dietary groups in terms of epididymal fat weight. This suggests that the tallow group had a larger fat pad than the sunflower oil group, but were also slightly heavier. Whilst differences in body weight were not significant, they appear to have been enough to account for the differences in fat depot.

3.4.3. Fatty Acid Content of Erythrocytes

Erythrocytes were analysed for fatty acid content as a method of assessing whether mice were consuming the diet. Fatty acid content of erythrocytes varied between dietary groups, and on the whole reflected the fatty acid content of the diet, suggesting that mice were eating the experimental diets and fatty acids metabolised by mice as would be expected. The tallow group had a similar EPA content of erythrocytes to the salmon oil erythrocytes, unlike the findings of the previous chapter. This may be due to the slightly

longer duration of the current study (48 days) compared to that of the previous chapter (37 days). Alternatively, it may be that the two different strains of mice used in these chapters respond differently to fatty acids. Similar EPA levels in the tallow and salmon oil groups may be because of the relatively high *n-3* and low *n-6* content of the tallow diet, despite the low total PUFA content, meaning that short chain *n-3* PUFAs were converted to long chain *n-3* PUFAs because of reduced competition from *n-6* PUFAs for the desaturase and elongase enzymes. This is also reflected in the high *n-3* to *n-6* ratio of erythrocytes in the tallow group. This is in accordance with the findings of other fatty acid feeding studies on fatty acid levels in plasma, serum and erythrocytes [70, 71, 79, 151-156].

3.4.4. Airway Responsiveness

There was no effect of diet on airway responsiveness. Whilst the effect of fish oils has not been examined in this model before, the effect of conjugated linoleic acid (CLA) has. Jaudszus *et al.* [181] and Kanwar *et al.* [182] fed *cis*-9, *trans*-11 CLA to ovalbumin-sensitised mice and showed a reduction in airway inflammation compared to a control group receiving diets containing sunflower oil [181] or soybean oil [182]. In humans, epidemiological studies have suggested that increased fish intake in childhood can reduce prevalence of asthma [183-186]. Nagakura *et al.* [187] supplemented asthmatic children with 300 mg fish oil capsules (84 mg EPA and 36 mg DHA) for 10 months and observed a reduction in asthma symptoms and an improvement in lung function compared to a control group receiving 300 mg olive oil.

3.4.5. Acute Skin Test

Ear swelling in response to ovalbumin in ovalbumin-sensitive mice is a measure of the immediate hypersensitivity response [175]. Ear swelling was lowest in animals receiving the salmon oil diet. Whilst several animal studies have examined the effect of fatty acids on delayed-type hypersensitivity [56, 89, 108, 188-190] or other measures of cell-mediated immunity [71, 75, 90], none have examined their effect on immediate hypersensitivity. Kull *et al.* [191] demonstrated a reduction in allergy and sensitisation to food and inhaled allergens in infants with a high fish consumption in the first year of life. Animals receiving the beef tallow diet showed the most ear swelling, which was significantly greater than seen in all other groups. Hwang *et al.* [192] demonstrated a positive correlation between saturated fatty acid content of erythrocytes and atopy, and a negative correlation between long chain *n-3* content and atopy in 4 – 6 year old children. Hoppu *et al.* [193] demonstrated a positive

relationship between saturated fatty acid intake of the pregnant woman and positive skin prick test of the child.

BALB/C and C57BL6 cell cultures produce different levels of T_H1 and T_H2 cytokines [127], and fish oil fatty acids may have different effects when added to cells cultured from these two strains of mice [194]. Addition of EPA was found to reduce IFN-γ production in cell cultures of BALB/C mice, but have no effect on IL-4 production, but in cell cultures of C57BL6 mice it had no effect of IFN-γ and reduced IL-4 production. In effect, EPA was further exaggerating the polarity of the dominance of the T_H cells; BALB/C cells became more T_H2 dominant, whilst C57BL6 cells had increased T_H1 dominance. It is important to note that these in vitro experiments were carried out using high concentrations of EPA (50 μM). The reduction in immediate hypersensitivity in BALB/C mice and lack of effect on cytokine production observed with fish oil feeding the this chapter is in contrast to the increase in T_H2 cytokines cells from BALB/C mice seen with EPA by May [194]. Perhaps differences may be observed in response to fatty acids when using different models.

In the previous chapter, ear swelling was measured 24 hour after injection with Influvac, and as such is a measure of delayed-type hypersensitivity (DTH). There, mice showed reduced ear swelling in the tallow, sunflower or linseed oil diets compared to those receiving the salmon oil diet. Immediate hypersensitivity is a T_H2 controlled response, and DTH is a T_H1 controlled response. These findings suggest that feeding salmon oil may reduce T_H2 responses, in both T_H1 and T_H2 dominant mice.

3.4.6. Antibody Production

There was no effect of diet on IgG1 or IgG2a concentration of plasma, but IgE concentration was lower in mice in the soybean oil group compared to the tallow group. T_H1 cells produce IFN-γ, which causes antibody isotype switching to IgG2a. T_H2 cells, however, produce IL-4, which causes isotype switching to IgE, which is the antibody responsible for immediate hypersensitivity and promotes IgG1 production. This suggests that the tallow diet may induce greater immediate hypersensitivity responses than the soybean oil diet. In contrast to the findings in the current study, Prickett *et al.* [195] found an increase in ova-specific IgE and IgG in ovalbumin-sensitive rats receiving a fish oil diet compared to those receiving a tallow diet. Duration of feeding was from body weight of 100 – 125 g to 225 – 250 g. Crevel *et al.* [107] found no difference between ova-specific IgE and IgG levels in ovalbumin-sensitive mice receiving PUFA or SFA rich diets for ten weeks. Chang *et al.* [196] demonstrated a reduction of anti-ovalbumin IgG1, but no effect on

anti-ovalbumin IgG2 or IgE in ovalbumin-sensitive female mice receiving ALA-rich (perilla oil) diet, compared to those on an LA-rich (corn oil) diet.

3.4.7. Cytokine Analysis

IL-4, IL-5 and IL-10 are produced by activated T_H2 cells; IL-4 causes naïve T-cells to differentiate into T_H2 cells, IL-5 increases production and activation of eosinophils, whilst IL-10 acts to inhibit activation and growth of T_H1 cells. IL-4 and IL-5 are also produced by activated mast cells. IFN-γ is produced by NK cells, T_H1 cells and CTLs; it is the signature cytokine of T_H1 cells, in much the same way that IL-4 is the signature cytokine of T_H2 cells. IFN-γ activates macrophages, causes isotype switching to opsonising and complement-fixing antibodies, and promotes differentiation of naïve T-cells to T_H1 cells, whilst inhibiting proliferation of T_H2 cells.

There was no effect of diet on IL-5 content of BALF, or IFN-γ or IL-10 production by splenocytes. CD3 stimulated IL-4 production by splenocytes in control and sunflower oil groups was lower than in linseed oil and tallow groups. Ovalbumin-stimulated IL-4 production by splenocytes did not differ between dietary groups. This suggests that fatty acids did not affect T_H1 / T_H2 cell balance, or eosinophil activation in ovalbumin-sensitive mice. Chang *et al.* [197] found a reduction in IFN-γ and IL-10, but no effect on IL-4 or IL-5 in BALF of ovalbumin-sensitive mice receiving a diet rich in ALA (perilla oil) compared to one rich in LA (corn oil) for 10 weeks. In the current study, only IL-5 was examined in BALF, whilst IFN-γ, IL-4 and IL-10 levels were determined in splenocytes. It may have been that if these cytokines were examined in BALF, a similar change would have been observed. In models in which mice were not sensitised to ovalbumin, fish oil and safflower oil diets, fed for 6 weeks, have been shown to reduce IFN-γ, but have no effect on IL-4 production by splenocytes, compared to a hydrogenated coconut oil diet [61]. Petursdottir and Hardardottir [198] found a reduction in IFN-γ, but no effect on IL-4 production by splenocytes in mice receiving fish oil compared to those corn oil diets for 6 weeks.

3.4.8. Histological Analysis

Cortex / medulla ratio was examined because as previous research at the University of Utrecht suggested that DHA may reduce medulla regions in the thymus (A. Hogenkamp, unpublished personal communication). T-cells mature in the thymus. The cortex contains the immature T-cells, which migrate to the medulla as they mature. Therefore, any effect on ratio of cortex to medulla may alter the area available for T-cell

maturation or may indicate an effect on maturation. However, the current study found no effect of diet on cortex / medulla ratio of mice thymuses.

3.4.9. Cell Analysis

Asthma is characterised by constriction of smooth muscle in the airway and an increase in mucosal exudation. This mucus includes inflammatory cells such as eosinophils, basophils, macrophages, neutrophils and lymphocytes. The mucus also contains epithelial cells. The sequence of events that occurs during asthma is as follows. Binding of IgE and T_H2 cells to mast cells causes their activation; this is the immediate-phase reaction. Cytokines produced by mast cells and T-cells, such as IL-4 and IL-5, cause the recruitment of eosinophils, basophils, neutrophils and more T_H2 cells; this is the late-phase reaction. Activated eosinophils, basophils and macrophages release lipid mediators such as prostaglandins and leukotrienes, which are responsible for inflammation resulting in airway constriction.

CD62L is a marker used for L-selectin expression and is responsible for the homing of lymphocytes to lymph nodes. CD62L is expressed on naïve lymphocytes, and its ligand on the high endothelial venules in the lymph node. Naïve lymphocytes adhere to the high endothelial venules in the lymph node, but since binding is weak, they role along the endothelial surface. This allows their circulation through the peripheral lymphatic system to secondary lymphoid tissues. CD62L is also expressed on neutrophils and monocytes, and allows their adherence to endothelium. Examining the level of CD62L expression, along with CD69 expression, a marker for activated leukocytes, gives an indication of the level of activated and naïve cells present in spleen. In addition, since blood cells generate from a common stem-cell progenitor, examining the balance of cells present in BALF and lymphoid tissue provides a means of assessing a mechanism of effect of fatty acids on asthma prevalence. However, there was no effect of diet on cells in BALF, or subsets of lymphocytes in spleens, thymuses or bone marrow. This is in agreement with the findings of the previous chapter, and section 1.4, where fatty acids were found not to affect the abundance of lymphocytes, T-cells, CTLs, T_H cells, B-cells, NK cells, monocytes, macrophages, and neutrophils. Hessel *et al.* [175] found no difference in cell abundance in ovalbumin-sensitive mice challenged with inhaled ovalbumin. In non-sensitised animals, Sanderson and Calder [199] found no effect of 8 weeks of feeding fish oil, hydrogenated coconut oil or safflower oil on CD62L expression on lymph node lymphocytes of rats. Zhang *et al.* [200] found no effect of fish oil on CD25+ expression compared to corn oil in mice receiving diets for 2 weeks.

3.4.10. Limitations

One limitation of this study is that a maximum of eight animals per diet group were studied; a larger sample size may have revealed stronger findings. A second limitation is that fatty acid composition data for immune cells were not available and erythrocyte fatty acids were used to examine the relationship between fatty acid status and immune response. Using data for immune cells would be more appropriate for this purpose. This study was carried out using male mice. It may be that a different effect would be observed in females, particularly in the linseed oil group, due to their increased efficiency to convert ALA to long chain *n-3* PUFAs [201]. This effect of gender on ALA conversion has been observed in humans[201], and more recently in rats[202], but has not yet been examined in mouse models. Measurements of airway hyper-responsiveness should only be viewed as an indication that there may not be any effect of fatty acids in this model, due to the fact that it is not an accurate measure of resistance [177].

3.4.11. Conclusions

The study set out to test hypotheses of reduced severity of allergic inflammation with salmon and linseed oils and increased severity with sunflower oil and tallow. These hypotheses were supported to some extent in that there was reduced immediate hypersensitivity in the salmon oil group and increased immediate hypersensitivity in the tallow group. However, varying the types of fatty acids in diets given to 3 to 4 week old mice for 48 days did not appear to affect airway responsiveness to ovalbumin. The increased ear swelling in animals on the tallow diet compared those receiving the soybean oil diet is reflected by an increase in IgE concentration in mice fed the tallow diet. Differences in IgE levels were not observed between other dietary groups. This may have been due to several factors, such as short feeding time (48 days) or age of the animals.

There did not appear to be changes induced in cell abundance in bronchoalveolar lavage fluid, or in cell subtypes in thymus, spleen and bone marrow, but these measurements do not determine the difference in balance of T_H1 / T_H2 cells. Examining the characteristic cytokines of these cells gave variable results. Splenocytes from mice receiving the tallow diet had increased IL-4 production in response to T-cell stimulation compared to control and sunflower oil groups, whilst there was no difference in response to ovalbumin stimulation. In addition, there was no difference observed in IFN-γ or IL-10 production in splenocytes, or IL-5 levels in BALF.

Thus, some of the diets (salmon oil, beef tallow) had effects on immediate hypersensitivity to ovalbumin in ovalbumin-sensitised mice. Beef tallow feeding also raised IgE in blood and elevated IL-4 production by anti-CD3 stimulated splenocytes. There was no effect of diet on lung function and salmon oil did not affect IgE or cytokine profiles. The diets were fed for 48 days beyond weaning and it may be that changing fatty acid availability earlier in life (i.e. pre-weaning) or a longer duration of feeding may have bigger effects. In the next two chapters studies in which mice were fed diets with varying fatty acid compositions during gestation and / or lactation are reported.

Chapter 4. Effect of Fatty Acids in Pregnancy on Markers of Immune Function in the Offspring

4.1. Introduction

Most studies on the effect of fatty acids on aspects of the immune response have been conducted in laboratory animals (rats, mice) post-weaning or on adult humans. However, it is possible that fatty acids may have a more pronounced effect on the immune system earlier in life. This is because significant development of the immune system occurs before and soon after birth. Furthermore, sensitisation to allergens has been shown to be present at birth indicating that those factors that lead to sensitisation are active in the foetus [132, 135]. However, whether an alteration in exposure to different fatty acids does influence early development and sensitisation of the immune system is not yet known. This study aims to explore this idea, and to determine the effect of variations in maternal fatty acid intake in pregnancy in the rat on immune parameters in the offspring.

4.1.1. Fatty Acids and Pregnancy

Fatty acids are of critical importance to foetal growth and development, because of their roles as components of membrane lipids, as regulators of gene expression, as eicosanoid precursors, and in cellular communication, and their direct interactions with proteins [203]. Exact mechanisms for fatty acid uptake across the placenta have not yet been fully clarified, but are thought to involve diffusion and membrane / cytosolic binding proteins including fatty acid binding protein (FABP), fatty acid transport protein (FATP) and fatty acid translocase (FAT) [204, 205]. FAT and FATP are integral proteins with membrane spanning regions, which may act as fatty acid translocators or transporters, whilst FABP is an extracellular (plasma membrane) binding protein, which is thought to act as an extracellular fatty acid acceptor [206]. These proteins show preferential uptake for particular fatty acids, favouring *n-3*and *n-6* PUFAs over non-essential fatty acids, and AA / DHA over LA / ALA [204, 205].

Fatty acids are taken up by the placenta in the form of NEFAs, either from NEFAs in maternal circulation, or as a product of maternal TAGs, broken down by placental lipase. Once in the cytoplasm of the syncytiotrophoblast, fatty acids are oxidised, used to form eicosanoids, or esterified, and then either stored or used. This is summarised in Figure 4-1.

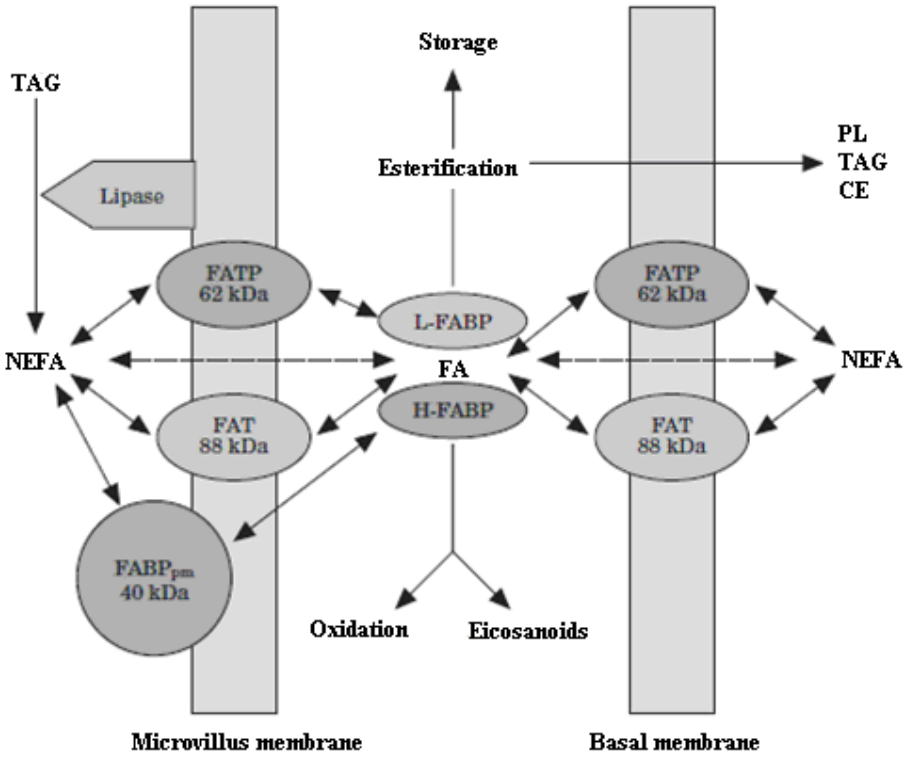

Figure 4-1: Fatty acid binding protein distribution on the microvillus / basal membranes, and within the cytoplasm of the syncytiotrophoblast (L-FABP, liver fatty acid binding protein; H-FABP, heart fatty acid binding protein. Both of these FABPs are named after the tissues in which they were first identified). Only NEFAs are taken up across the plasma membrane, and these may be derived directly from the maternal circulation, or from TAGs after the actions of placental lipases. Export of PL, CE and TAG into foetal circulation has been suggested [207]. Taken from Haggarty [206].

Long chain PUFA content is higher in the foetal blood than maternal blood [204, 205]. Explanations for this include the selective transport of these fatty acids by the placenta, preferential accumulation of long chain PUFAs into esterified lipids, selective lipolysis of long chain PUFAs in maternal circulating TAGs by placental lipase, or increased mobilisation of maternal adipose tissues during late gestation. Long chain PUFAs can either be provided preformed from the mother, or synthesised from the shorter chain *n-3* and *n-6* PUFAs derived from the mother. Studies have demonstrated, however, that preformed DHA is much more effectively stored in tissues as DHA than DHA produced from ALA [208-212]. This may well be due to the fact Δ-5 and Δ-6 desaturases, although present in foetal liver from early in gestation, have low activity before birth [213].

In addition, expression of genes involved in fatty acid uptake, differentiation of the placental trophoblast, and production of human chorionic gonadotrophin are affected by maternal fatty acids; hence, maternal fatty acids may not only affect placental transport, but also placental biology [205].

4.1.2. Fatty Acids in Pregnancy and the Immune System

Studies have suggested that stimuli or insults during "critical" periods of development in pregnancy may cause short-term survival adaptations in pregnancy, which in turn may result in development of diseases during child- or adult-hood (reviewed by Gluckman and Hanson [129, 214]). These critical periods may coincide with phases of rapid cell growth [215]. This long-lasting effect of *in utero* conditions is known as "foetal programming" or the "Barker hypothesis". The later name exists as research into this phenomenon originated from a paper by Barker *et al.* [128], in which low birth weight was linked with high risk of development of ischemic heart disease in adulthood.

Following on from this, it is possible that fatty acids in pregnancy could have long lasting effects on the immune system because of their effects on gene regulation influencing the development and maturation of the immune system. This effect of fatty acids may be even more pronounced perinatally than in adulthood as significant development of the immune system occurs before birth [216]. Furthermore sensitisation to allergens has been shown to be present at birth indicating that those factors that lead to sensitisation are active in the foetus [132, 135]. However, whether an alteration in exposure to different fatty acids influences early development and sensitisation of the immune system is not yet known. Recent research has shown that the source of fat in the diet of pregnant rats has the potential to alter the immune system of offspring at around the time of birth; specifically feeding a diet rich in salmon oil diet increased the proportion of CTL and NK in the foetal thymus [133, 217, 218]. However, whether this change is maintained into later life remains to be determined as this has not been explored previously.

4.1.3. Aims of the study

The study aims to investigate the effect of maternal diets differing in fatty acid composition, including different levels and different types of *n-3* and *n-6* fatty acids, on markers of immune status and function in the offspring of rats, in particular the types of lymphocytes in blood and lymphoid organs. Altering maternal fatty acid intake may cause structural changes in foetal and/or maternal immune cells, which in turn may affect immune function. The study aims to determine where, in this pathway, change occurs, and whether these differences in immune function are maintained later in life. The study is summarised in Figure 4-2.

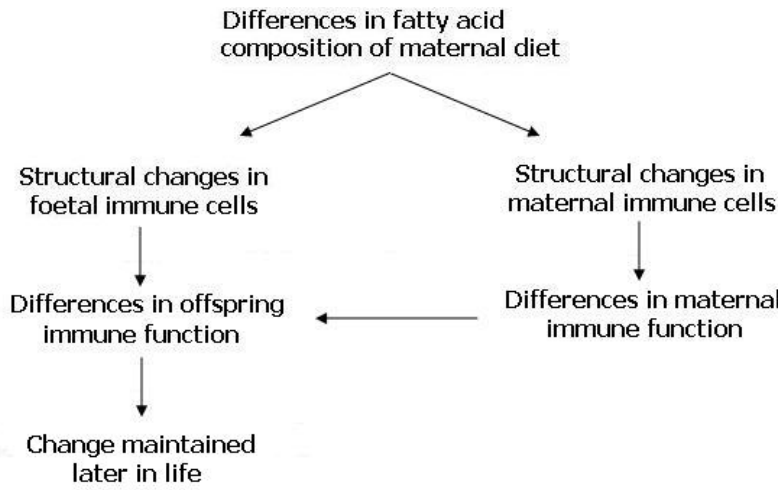

Figure 4-2: Causal chain linking altered maternal fatty acid intake to altered offspring immune function. Changes in fatty acid composition of the maternal diet may cause changes in structure of foetal and / or maternal immune cells. This in turn may alter their function.

In order to achieve these aims, three experimental diets will be fed to dams from conception until birth; chow will be fed thereafter. One diet will contain salmon oil as the fat source; this diet is high in long chain *n-3* fatty acids. The second diet will contain soybean oil as the fat source; this diet contains a balance of plant *n-3* and *n-6* fatty acids. The third diet will contain sunflower oil as the fat source; this diet is rich in *n-6* fatty acids. These three experimental diets are high fat (13 %). Dams will be transferred to chow, a low fat (3 %) diet after birth, and offspring weaned onto chow at 3 weeks of age.

4.1.4. Hypotheses

The study set out to test the following hypotheses:

- Feeding the salmon oil diet in pregnancy will result in increased levels of long chain *n-3* PUFAs in maternal plasma and tissues. Long chain *n-3* PUFAs will be present at high levels in the plasma of offspring of the salmon oil group at 3 weeks, but the level will decrease with time
- Feeding the soybean oil diet in pregnancy will cause a balance of short chain *n-3* and *n-6* PUFAs in plasma of dams and offspring, at higher levels than the long chain *n-3* and *n-6* PUFAs. Level of short chain *n-3* and *n-6* PUFAs in offspring plasma will not be affected by time as offspring are weaned onto chow whose fat source is soybean oil (albeit at a lower concentration)
- Feeding the sunflower oil diet in pregnancy will result in an increased level of *n-6* PUFAs in plasma of dams, and offspring at 3 weeks, but level of *n-6* PUFAs in offspring plasma will decrease with time

- Immune cell abundance of offspring blood and lymphoid organs will be affected by altering the fatty acid composition of the diet pregnant rats: specifically offspring in the salmon oil group will have increased numbers of CTLs and NK cells.

4.2. Materials and Methods

4.2.1. Animals, Diets and Materials

4.2.1.1. Animals

Male and female Wistar rats were obtained from the Biomedical Research Facility, University of Southampton (Southampton, UK).

4.2.1.2. Diets

The high-fat experimental diets (sunflower oil, salmon oil and soybean oil) and low-fat soybean oil-based chow (CRM-1) were the same as those used previously (2.2.1.2).

4.2.1.3. Chemicals

Heparin was from CP Pharmaceuticals Limited (Wrexham, Flintshire, UK). L-Glutamine, streptomycin / penicillin, and RPMI-1640 (without L-glutamine) were from PAA Laboratories Limited (Yeovil, Somerset, UK). Phosphate buffered saline (PBS) was from Oxoid Limited (Basingstoke, Hampshire, UK). Coulter Isoton Diluent II was from Beckman Coulter (UK) Limited (High Wycombe, Buckinghamshire, UK). Foetal calf serum (FCS), concanavalin A (Con A), histopaque (density 1.077 g/ml), bovine serum albumin (BSA), formaldehyde, sodium azide and all standard laboratory reagents were from Sigma-Aldrich Limited (Gillingham, Dorset, UK). FACs lysing solution was from BD Biosciences (Cowley, Oxford, UK). The following antibodies were from AbD Serotec (Kiddlington, Oxford, UK):

- Fluorescein isothiocyanate (FITC)-labelled mouse anti-human IgG1,
- FITC-labelled mouse anti-human IgM,
- r-phycoerythrein (RPE)-labelled mouse anti-human IgG1,
- FITC-labelled mouse anti-rat CD163 (IgG1),
- FITC-labelled mouse anti-rat CD161 (IgG1),
- FITC-labelled mouse anti-rat CD3 (IgM) / RPE-labelled mouse anti-rat CD8 (IgG1)
- FITC-labelled mouse anti-rat CD3 FITC (IgM) / RPE-labelled mouse anti-rat CD4 RPE (IgG1),
- FITC-labelled mouse anti-rat CD3 FITC (IgM) / RPE-labelled mouse anti-rat CD45RA RPE (IgG1).

4.2.2. Methods

An overview of the trial is shown in Figure 4-3. Eighteen 10-week old nulliparous Wistar rats were bred with six age-matched males. The presence of a vaginal plug was used as an indicator of potential conception and birth of offspring was designated day 0 of postpartum development. Following conception, animals were randomised into groups to receive one of the three experimental diets. Post-parturition, all animals were maintained on chow. Body weight of dams was measured weekly. At 2-7 days of age, litters were culled to 8 offspring (4 males, 4 females), in order to minimise variation in milk composition, milk yield, offspring nutrient intake and behavioural activities of offspring and dams [219, 220]. Post-parturition, offspring remained with dams until weaning at day 21. Pregnant dams and weaned offspring were housed separately in shoebox cages, and kept at 21 ± 2 °C, under a 12 hour light : 12 hour dark cycle. At all times, animals had *ad libitum* access to food and water. All studies were carried out in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, under project licence number 30/1889, and personal license number 70/20024.

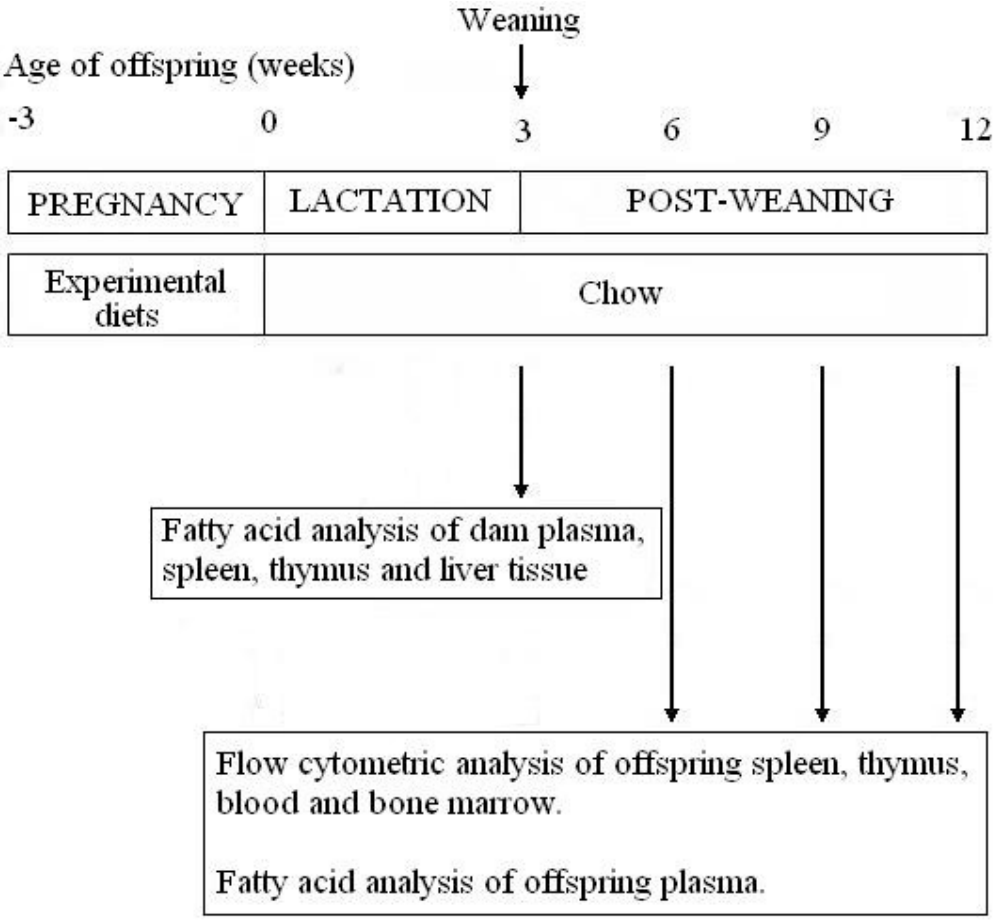

Figure 4-3: Schematic diagram of trial showing the timing of feeding and of sample collection and the outcomes measured.

4.2.2.1. Fatty Acid Analysis of Diets and Maternal Plasma

To extract lipids, 100 mg of diet was homogenised in 0.8 ml of 0.9 % NaCl in triplicate. Samples of plasma were adjusted to 0.8 ml with 0.9 % NaCl, if 0.8 ml sample volume of plasma was not available. Internal standards were added at levels listed in Table 4-1, and samples vortexed.

Table 4-1: Internal standards used for FA analysis of corresponding lipid classes. Taken from Burdge et al. [221].

Lipid class	Numerical formula of standard fatty acid	Systematic name of standard	Amount used in feed analysis (µg)	Amount used in plasma (µg)
Phosphatidylcholine	15:0	Dipentadecanoyl PC	n/a	100
Phosphatidylethanolamine	17:0	Diheptadecanoyl PE	n/a	2
Non-esterified fatty acids	21:0	Heneicosanoic acid	100	25
Cholesteryl esters	17:0	Cholesteryl heptadecanoate	n/a	500
Triacylglycerol	15:0	Tripentadecanoin	n/a	150

Lipid classes were separated by solid phase extraction, using the method of Burdge *et al.* [221]. Briefly, samples were dissolved in 1 ml dry chloroform, vortexed and allowed to drip through an aminopropylsilica solid phase extraction cartridge under gravity. The column was washed with 2 ml dry chloroform, and the chloroform washes (which contained TAG and CE were dried under nitrogen at 40°. PC was eluted by the addition of 2ml dry choloroform / methanol (60:40, v/v) under vacuum, and dried under nitrogen at 40°. NEFA was collected by the addition of 2 ml chloroform / methanol / glacial acetic acid (100 : 2 : 2, v/v/v) under vacuum, and dried under nitrogen at 40 °. 4 ml of dry hexane was added to fresh aminopropylsilica solid phase extraction cartridge. The TAG / CE elucidate was dissolved in 1 ml dry hexane and then added to this cartridge. CE was eluted by the addition of 2 ml dry hexane, and dried under nitrogen at 40 °. TAG was eluted by the addition of 2ml dry hexane / chloroform / ethyl acetate (100 : 5 : 5 v/v/v) under vacuum, and samples dried under nitrogen at 40 °.

Fatty acid methyl esters of these lipid fractions were prepared, and samples analysed using the method described previously (2.2.2.3).

4.2.2.2. Preparation of Immune Cells from Lymphoid Organs

3-, 6-, 9-, or 12-week old animals were euthanized by carbon dioxide inhalation and cervical dislocation. Two pups from each dam (one male, one female) were pooled to create one sample, in order to increase amount of tissue / blood available and reduce the effect of gender. Blood was collected into heparinised tubes by cardiac puncture, and placed on ice. The thymus, spleen, and both tibia and femurs were removed and immediately placed into RPMI culture medium on ice.

Approximately 1 ml of the blood sample was decanted into an eppendorf, and stored at 4°C until required for flow cytometry. Bone marrow cells were collected by cutting between the metaphysis and diaphysis at both ends of the bone, and flushing out bone marrow using a 21G x 1 1/2" needle and 10 ml syringe containing RPMI. These cells, together with thymuses and spleens were pushed through a tea strainer to break up the tissue and to release cells. Cells were then filtered using a lens tissue to remove debris, and centrifuged at 1000 rpm for ten minutes. The supernatant was removed, and pelleted cells resuspended using RPMI. Cells were carefully layered onto 10 ml histopaque (density 1.077 g/ml), and centrifuged at 2000 rpm for 15 minutes at 15 °C, without a brake. Mononuclear cells were removed into a fresh tube, to which RPMI was added, before being centrifuged at 1500 rpm for 7 minutes. The supernatant was removed, and the pellet resuspended in 1 ml RPMI.

4.2.2.3. Determination of Cell Numbers in Lymphoid Tissues

10 μl of the cell suspension from the thymus, spleen and bone marrow samples were placed in 10 ml isoton, and cells counted using a Beckman Z1 Coulter Counter (High Wycombe, UK). This was repeated, values summed together and multiplied by 1000, to determine the cell count per 1 ml suspension.

4.2.2.4. Determination of Cell Subsets in Lymphoid Tissues using Flow Cytometry

Thymus, spleen and bone marrow cells were diluted in RPMI (if necessary) to form a suspension of 1 x 10^7 cells/ml. Whole blood was not diluted. Eight tubes were set up, one for each of the antibody stains or controls listed in Table 4-2. A ninth tube was set up for splenic cells, which was used to identify splenic macrophages.

Table 4-2: Antibodies used for FACs analysis. All antibodies were mouse anti-rat.

Antibody	Cell type identified	Spleen	Thymus	Bone Marrow
Blank	(Control)	X	X	X
FITC Anti-IgG_1	(Control)	X	X	X
FITC Anti-IgM	(Control)	X	X	X
RPE Anti-IgG_1	(Control)	X	X	X
FITC (IgG_1) Anti-CD161	NK cells	X	X	X
FITC (IgM) Anti-CD3 / RPE (IgG_1) Anti-CD8	CTLs	X	X	X
FITC (IgM) Anti-CD3 / RPE (IgG_1) Anti-CD4	T_H cells	X	X	X
FITC (IgM) Anti-CD3 / RPE (IgG_1) Anti-CD45RA	B-cells	X	X	X
FITC (IgG_1) Anti-CD163	Splenic macrophages	X	-	-

10 µl of each fluorescently labelled antibody was added to 100 µl of cell suspension or blood, and then vortexed. Samples were covered in parafilm, and incubated at room temperature for 30 minutes. 2 ml FACs lysing solution was added to whole blood samples, which were then vortexed, covered again, and incubated at 4 °C for a further 10 minutes. All tubes were centrifuged at 1000 rpm for seven minutes, following which the supernatant was discarded. 2 ml mPBS (PBS containing 1 g/l bovine serum albumin and 0.65 g/l sodium azide) was added, and samples vortexed before centrifugation at 1000 rpm for seven minutes, to wash cells. The supernatant was discarded, and the washing step repeated. Cells were suspended in 200 µl FACs fix (PBS containing 2 % formaldehyde), and vortexed. Samples were stored at 4 °C, until analysis, which took place within 24 hours. Cell types were analysed using a Becton Dickinson FACSCalibur flow cytometer (Cowley, Oxford, UK) and CellQuest software, which recorded 10^4 events per sample.

4.2.2.5. Statistical Analysis

Results were analysed using SPSS version 15.0. Parameters were analysed using Levene's test of homogeneity to ensure groups had equal variances, in which case they were compared using one-way analysis of variance (ANOVA), followed by *post-hoc* testing (Tukey). If Levene's test showed unequal variances to be present, groups were compared using the Kruskal-Wallis test. Where this was used, it is reported in the results section. If the Kruskal-Wallis test indicated a significant effect, Mann-Whitney U independent group comparisons tests were then carried out to compare each combination of groups.

4.3. Results

4.3.1. Gestational Data of Rats fed the Different Diets

There were no significant differences among the dietary groups in terms of weight at conception or at days 7, 14 or 20 of gestation, number of offspring or gestation length (Table 4-3).

Table 4-3: Weights of dams at conception and at days 7, 14 and 20 of pregnancy, number of offspring, and length of gestation in the different dietary groups. Results are shown as mean ± SEM. There were no statistical differences among groups for any outcome (p>0.05).

	Salmon oil (n=11)	Soybean oil (n=9)	Sunflower oil (n=11)
Weight at conception (g)	211.7 ± 3.9	214.9 ± 3.2	214.4 ± 3.8
Weight at day 7 of gestation (g)	227.8 ± 3.5	233.7 ± 5.0	233.8 ± 3.9
Weight at day 14 of gestation (g)	254.6 ± 4.5	258.9 ± 4.6	258.0 ± 4.2
Weight at day 20 of gestation (g)	311.6 ± 6.5	308.8 ± 7.6	316.6 ± 6.1
Number of offspring	12 ± 1	10 ± 1	11 ± 1
Length of gestation (days)	21.4 ± 0.2	21.1 ± 0.3	21.7 ± 0.2

4.3.2. Body Weights of Offspring of Rats fed the Different Diets

Body weights of male and female offspring are shown in Table 4-4. There was no difference in male or female body weight at any time point as a result of maternal diet. Across all diets, body weights increased with time, but there was no difference among dietary groups.

Table 4-4: Bodyweights of offspring at different time points after weaning from dams that received different diets during pregnancy. There were no statistical differences among groups for either gender (p>0.05).

Gender	Week		Salmon oil	Soybean oil	Sunflower oil
Female	3	n	11	7	9
		Mean ± SEM	44.3 ± 5.9	48.0 ± 8.7	44.8 ± 6.9
	6	n	8	7	9
		Mean ± SEM	125.8 ± 2.5	115.3 ± 5.3	122.1 ± 7.4
	9	n	12	11	12
		Mean ± SEM	169.7 ± 3.0	173.1 ± 3.8	178.3 ± 4.7
	12	n	10	10	12
		Mean ± SEM	205.6 ± 6.0	199.6 ± 5.3	212.6 ± 3.9
Male	3	n	11	9	13
		Mean ± SEM	46.5 ± 6.2	49.0 ± 6.7	39.8 ± 5.4
	6	n	10	5	9
		Mean ± SEM	146.0 ± 7.4	155.8 ± 13.6	144.4 ± 8.4
	9	n	12	9	12
		Mean ± SEM	247.4 ± 7.3	256.2 ± 10.1	249.0 ± 6.4
	12	n	9	7	10
		Mean ± SEM	323.3 ± 7.8	330.9 ± 14.2	329.1 ± 5.6

4.3.3. Tissue Weights of Offspring of Rats fed the Different Diets

Spleen and thymus weights of offspring are shown in Table 4-5 and Table 4-6 respectively. There was no effect of diet at any time point, although there was an effect of time. Spleens of females weighed less at week 3 than at other time points, but there was no significant difference in weight in weeks 6 to 9. In males, spleens increased in weight up until week 9. Thymus weights were significantly greater at weeks 6, 9 and 12 than at week 3 in females and males of all groups.

Table 4-5: Spleen weights of male and female offspring at different stages after birth (3, 6, 9 and 12 weeks of age). Results are shown as mean ± SEM. Values with different letters denote differences in spleen weight in dietary groups between time points ($p<0.05$).

	Week		Salmon oil	Soybean oil	Sunflower oil
Female spleen weight (g)	3	n	11	7	9
		Mean±SEM	0.19 ± 0.02^{a}	0.22 ± 0.03^{a}	0.19 ± 0.02^{a}
	6	n	8	7	9
		Mean±SEM	0.43 ± 0.04^{b}	0.38 ± 0.03^{b}	0.38 ± 0.03^{b}
	9	n	12	11	12
		Mean±SEM	0.45 ± 0.02^{b}	0.47 ± 0.02^{b}	0.45 ± 0.01^{b}
	12	n	10	10	12
		Mean±SEM	0.52 ± 0.02^{b}	0.52 ± 0.01^{b}	0.50 ± 0.02^{b}
Male spleen weight (g)	3	n	11	9	13
		Mean±SEM	0.20 ± 0.02^{a}	0.20 ± 0.02^{a}	0.17 ± 0.02^{a}
	6	n	10	5	8
		Mean±SEM	0.52 ± 0.03^{b}	0.51 ± 0.05^{b}	0.48 ± 0.03^{b}
	9	n	12	9	12
		Mean±SEM	0.65 ± 0.03^{bc}	0.60 ± 0.04^{b}	0.62 ± 0.02^{c}
	12	n	9	7	10
		Mean±SEM	0.72 ± 0.02^{c}	0.69 ± 0.04^{b}	0.70 ± 0.02^{c}

Table 4-6: Thymus weights of male and female offspring at different stages after birth (3, 6, 9 and 12 weeks of age). Results are shown as mean ± SEM. Values with different letters denote differences in thymus weight in dietary groups between time points ($p<0.05$).

	Week		Salmon oil	Soybean oil	Sunflower oil
Female thymus weight (g)	3	n	11	7	9
		Mean±SEM	0.26 ± 0.03^{a}	0.25 ± 0.04^{a}	0.26 ± 0.04^{a}
	6	n	8	7	9
		Mean±SEM	0.56 ± 0.02^{b}	0.50 ± 0.04^{b}	0.55 ± 0.04^{b}
	9	n	12	11	12
		Mean±SEM	0.59 ± 0.02^{b}	0.59 ± 0.04^{b}	0.58 ± 0.02^{b}
	12	n	10	10	11
		Mean±SEM	0.58 ± 0.03^{b}	0.58 ± 0.03^{b}	0.56 ± 0.03^{b}
Male thymus weight (g)	3	n	11	9	13
		Mean±SEM	0.23 ± 0.02^{a}	0.28 ± 0.04^{a}	0.23 ± 0.02^{a}
	6	n	10	5	9
		Mean±SEM	0.57 ± 0.03^{b}	0.61 ± 0.04^{b}	0.55 ± 0.04^{b}
	9	n	12	9	12
		Mean±SEM	0.69 ± 0.03^{b}	0.69 ± 0.04^{b}	0.70 ± 0.02^{b}
	12	n	9	7	10
		Mean±SEM	0.65 ± 0.02^{b}	0.66 ± 0.03^{b}	0.66 ± 0.04^{b}

Thymus, spleen and body weights of offspring aged 3, 6, 9 and 12 weeks are shown graphically in Figure 4-4. These plots clearly demonstrate the lack of differences among dietary groups at any time point.

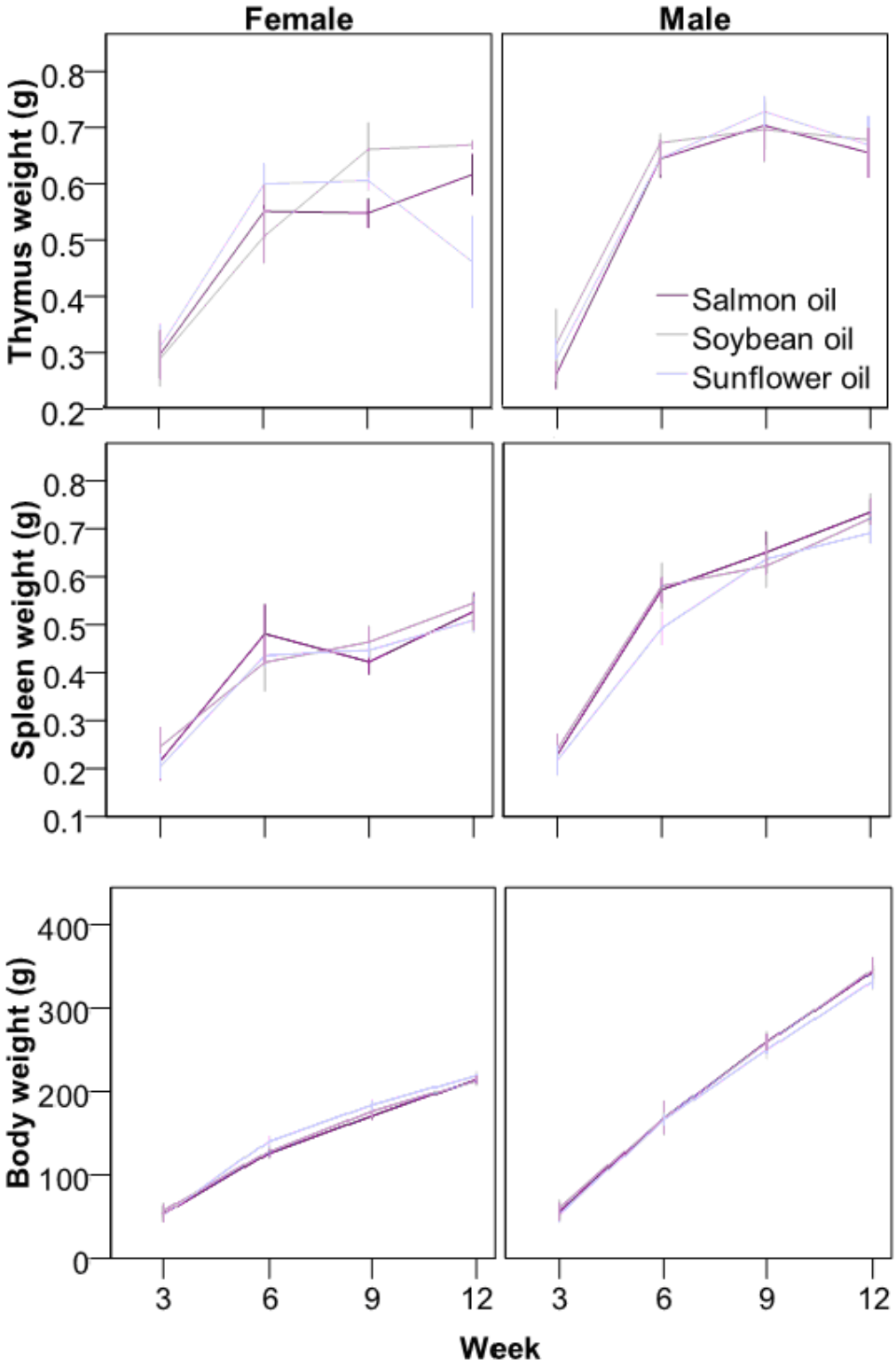

Figure 4-4: Thymus (top), spleen (middle) and body (bottom) weights over time.

4.3.4. Fatty Acid Analysis of Plasma of Rats fed the Different Diets

Maternal plasma fatty acid profile is shown in detail in Appendix Three. Fatty acids of interest are shown in Figure 4-5. 18:2 *n-6* content of CE was higher in the salmon oil group than the sunflower oil group. 20:5 *n-3* and total *n-3* contents and ratio of *n-3* to *n-6* PUFAs in the CE fraction were higher in the salmon oil group than the soybean and sunflower oil groups. There was no difference in fatty acid profile in the NEFA fraction between dietary groups. This is as would be expected, since the NEFA fraction partly represents recently absorbed dietary fatty acids; dams from all groups had been receiving chow since parturition, three weeks previously. *Trans* 18:1 *n-9* in the PC fraction of maternal plasma were highest, and 18:3 *n-6* and 20:3 *n-6* levels were lowest in the in the soybean oil group compared to other dietary groups. 20:5 *n-3* levels were higher in the salmon oil group compared to the soybean oil group. *Trans* 18:1 *n-9* levels in the TAG fraction were higher in the soybean oil group than the sunflower oil group.

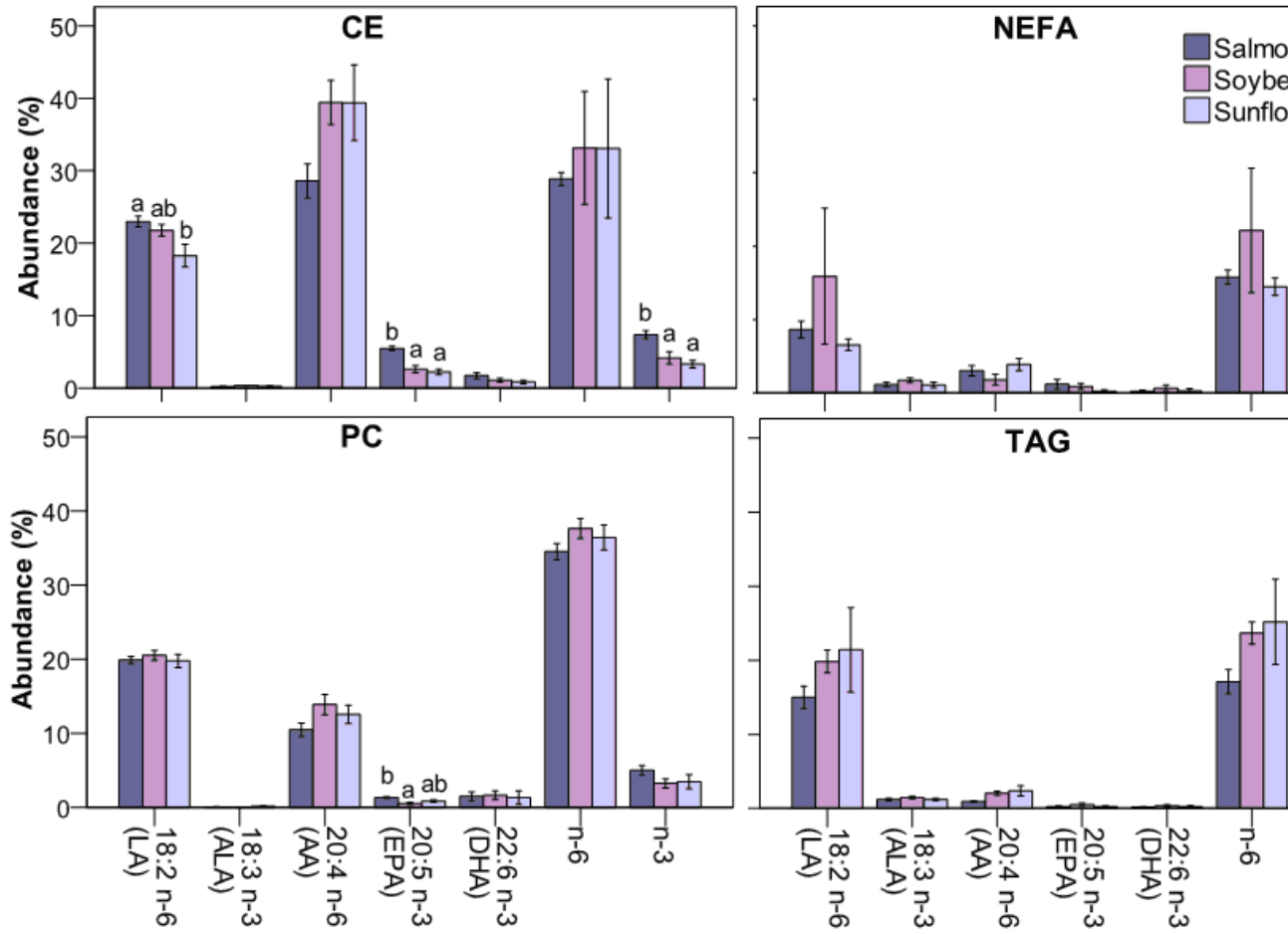

Figure 4-5: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents of maternal plasma, three weeks after parturition and
Values with different letters denote differences in fatty acid abundance between dietary groups (p<0.0

4.3.5. Fatty Acid Analysis of Tissues of Rats fed the Different Diets

Maternal plasma fatty acid profile of liver, spleen and thymus is shown in detail in Appendix Three. Fatty acids of interest are shown in Figure 4-6 (liver), Figure 4-7 (spleen) and Figure 4-8 (thymus).

Out of the fatty acids of interest, only the PC and PE fractions showed any differences between groups in the liver. In the PC fraction, AA content was higher in the sunflower oil group than the salmon oil group, and EPA, DHA and total *n-3* contents were highest in the salmon oil group compared to other groups, whilst total *n-6* content was lowest in this group compared to others. In the PE fraction, DHA and total *n-3* contents were highest in the salmon oil group.

In the spleen, differences were seen in NEFA fraction in addition to PC and PE. In NEFA, DHA and total *n-3* contents were highest, whilst total *n-6* content was lowest in the salmon oil group compared to both other groups. In the PC fraction of spleen tissue, LA, DHA and total *n-3* content was higher in the salmon oil group compared to the sunflower oil group; the soybean oil group was not different for these fatty acids from either salmon oil or sunflower oil groups. The PE fraction of spleen showed a higher AA content in the sunflower oil group compared to the salmon oil group, whilst AA content of soybean oil group was not different from either other group. EPA content of the salmon oil group was higher than the soybean oil group in the same fraction. Also, DHA and *n-3* contents were higher, whilst *n-6* content was lowest in the salmon oil group compared to both soybean oil and sunflower oil groups.

Differences in fatty acid content were observed in the same three lipid fractions in the thymus. In NEFA, DHA and total *n-3* contents were highest in the salmon oil group compared to both other groups, which were not different from one another. AA content in the PC fraction was higher in the sunflower oil group than the salmon oil group, whilst soybean oil was not different from either other group. In the PE fraction of the thymus, EPA content was higher in the salmon oil group than the sunflower oil group; soybean oil EPA content of thymus was not different from the salmon or sunflower oil groups. DHA and *n-3* contents of the salmon oil group were higher than that of the soybean oil and sunflower oil groups, which did not show statistical difference from one another.

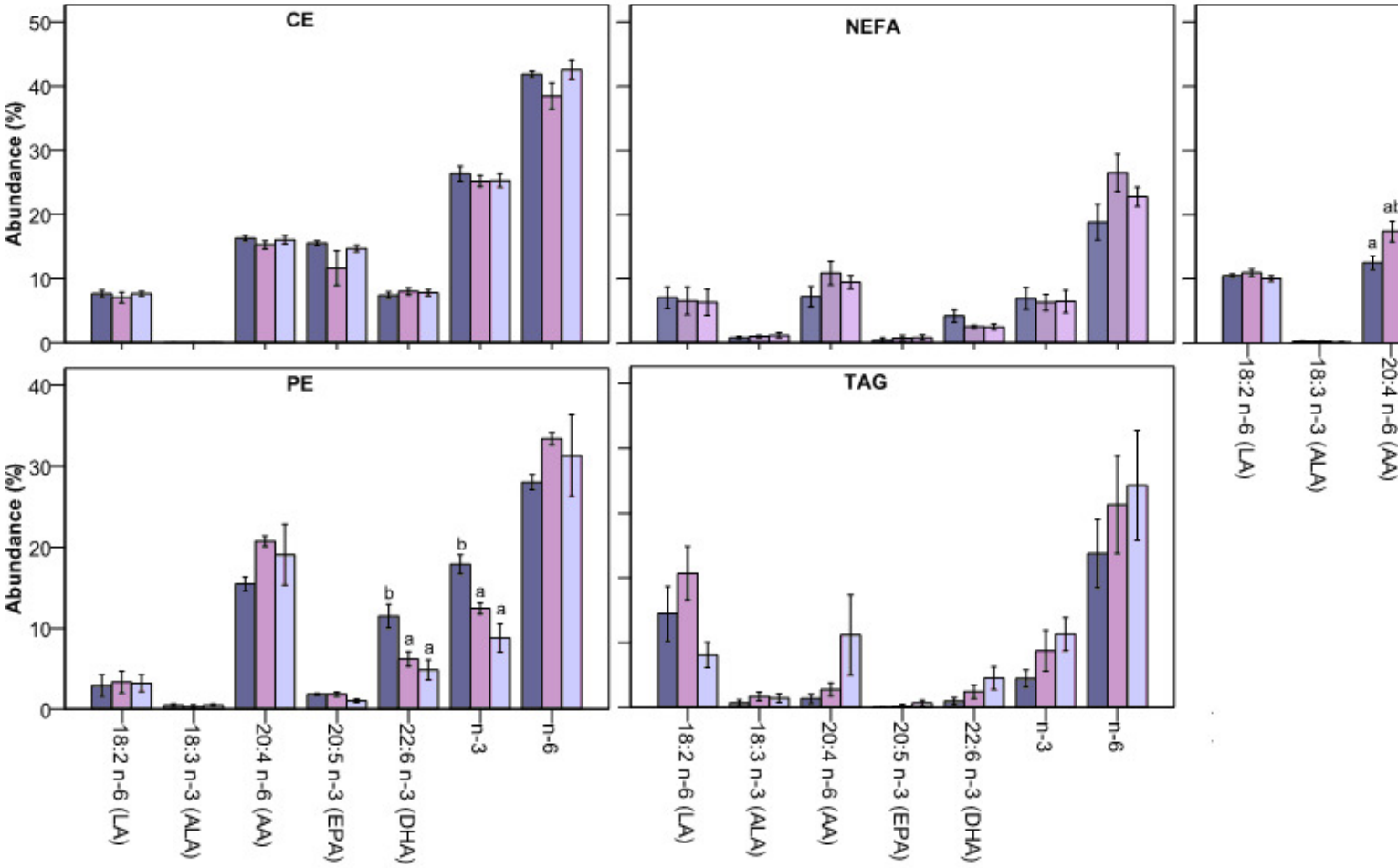

Figure 4-6: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents of maternal liver, three weeks after parturition and cl
Values with different letters denote differences in fatty acid abundance between dietary groups (p<0.0

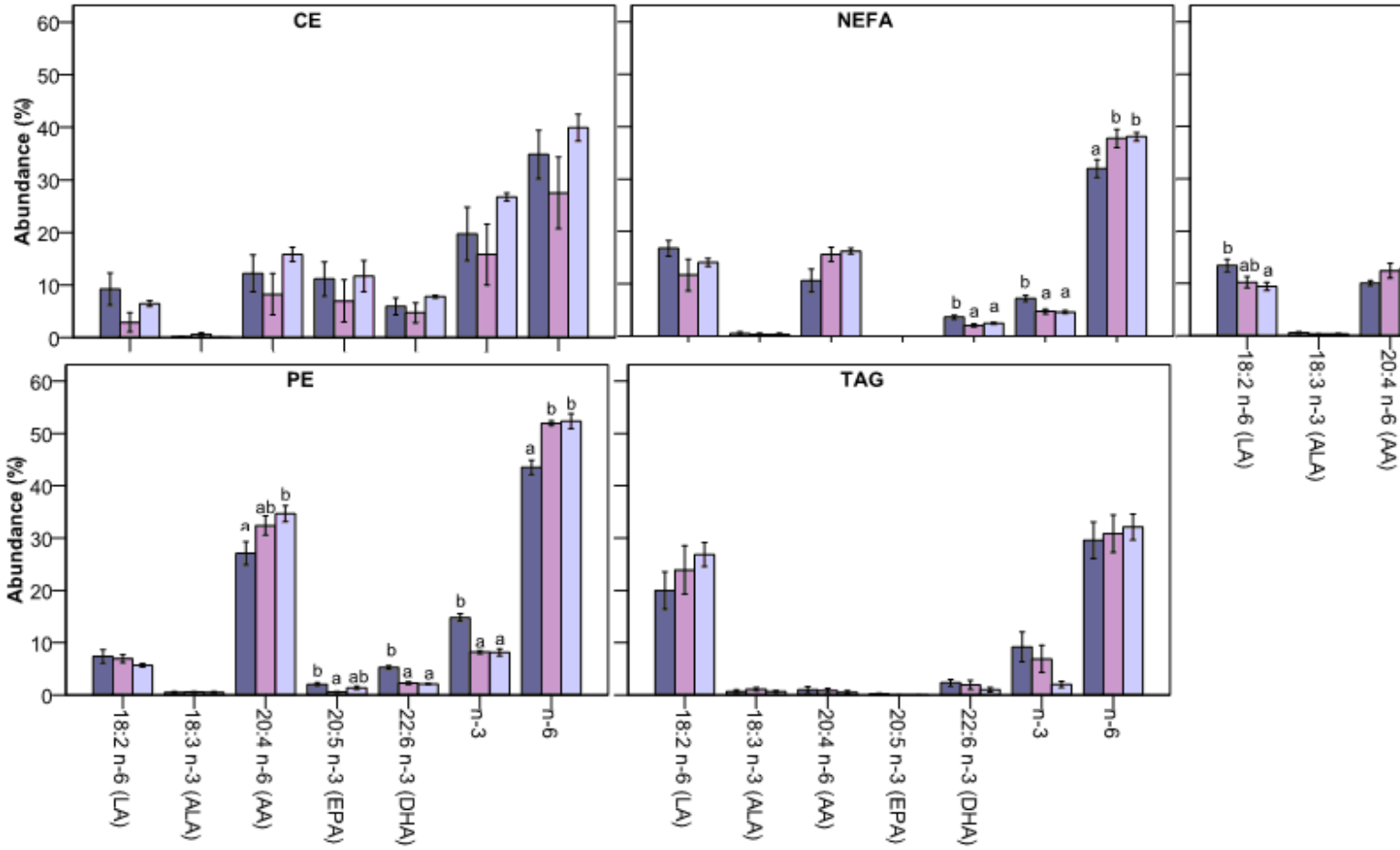

Figure 4-7: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents of maternal spleen, three weeks after parturition and c
Values with different letters denote differences in fatty acid abundance between dietary groups (p<0.0

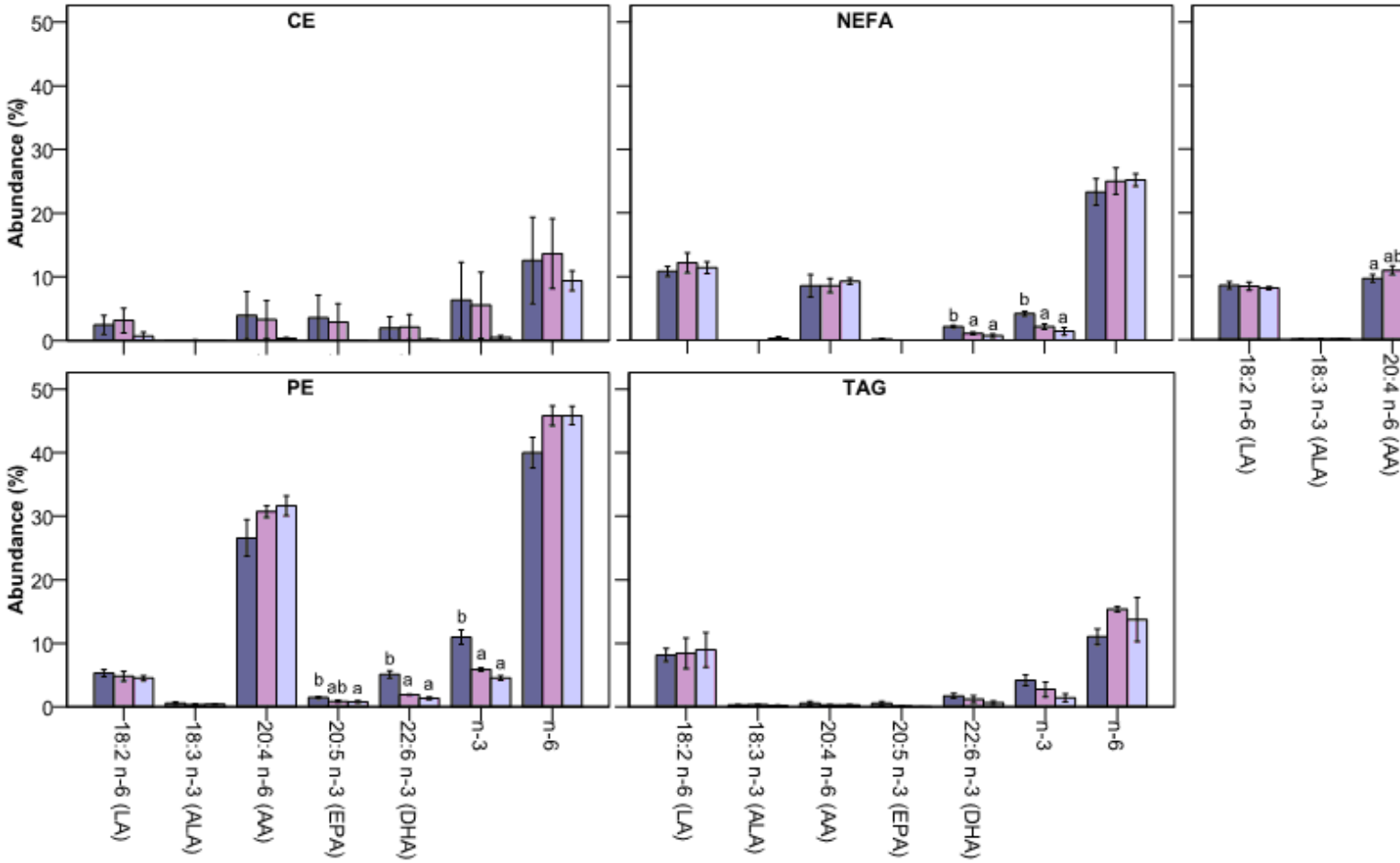

Figure 4-8: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents of maternal thymus, three weeks after parturition and
Values with different letters denote differences in fatty acid abundance between dietary groups (p<0.0

4.3.6. Fatty Acid Analysis of Plasma from Offspring of Rats fed the Different Diets

Offspring plasma fatty acid profile is shown in detail in Appendix Three. Fatty acids of interest are shown in Figure 4-9 to Figure 4-12. In all cases there was no effect of diet, but there was an effect of time. Total *n-6* content of the CE fraction was lower at week 3 than week 6 in the sunflower oil group.

In the NEFA fraction, LA content was lowest at week 3 in the salmon oil and sunflower oil groups compared to all other groups. In the soybean oil group, LA content was lower at week 3 than at week 6. AA content was lower at week 3 than week 12 in the salmon oil group; in the sunflower oil group it was lower at week 3 than all other weeks. Total *n-3* content was lower at week 3 than week 6 in the soybean oil group. Total *n-6* content was lower at week 9 than week 3 in the salmon oil group, and highest at week 3 than weeks 9 and 12 in the sunflower oil group.

In the PC fraction, AA content was lowest at week 3 than all other weeks in the salmon oil group. EPA content was highest at week 6 than all other weeks in the sunflower oil group.

In the TAG fraction, LA content was lowest at week 3 compared to other weeks in the salmon oil group; in the sunflower oil group, LA content was lower at week 3 than week 6. ALA content was lower at week 3 than week 12 in the sunflower oil group. Total *n-6* content was higher at week 9 than week 3 in the salmon oil group.

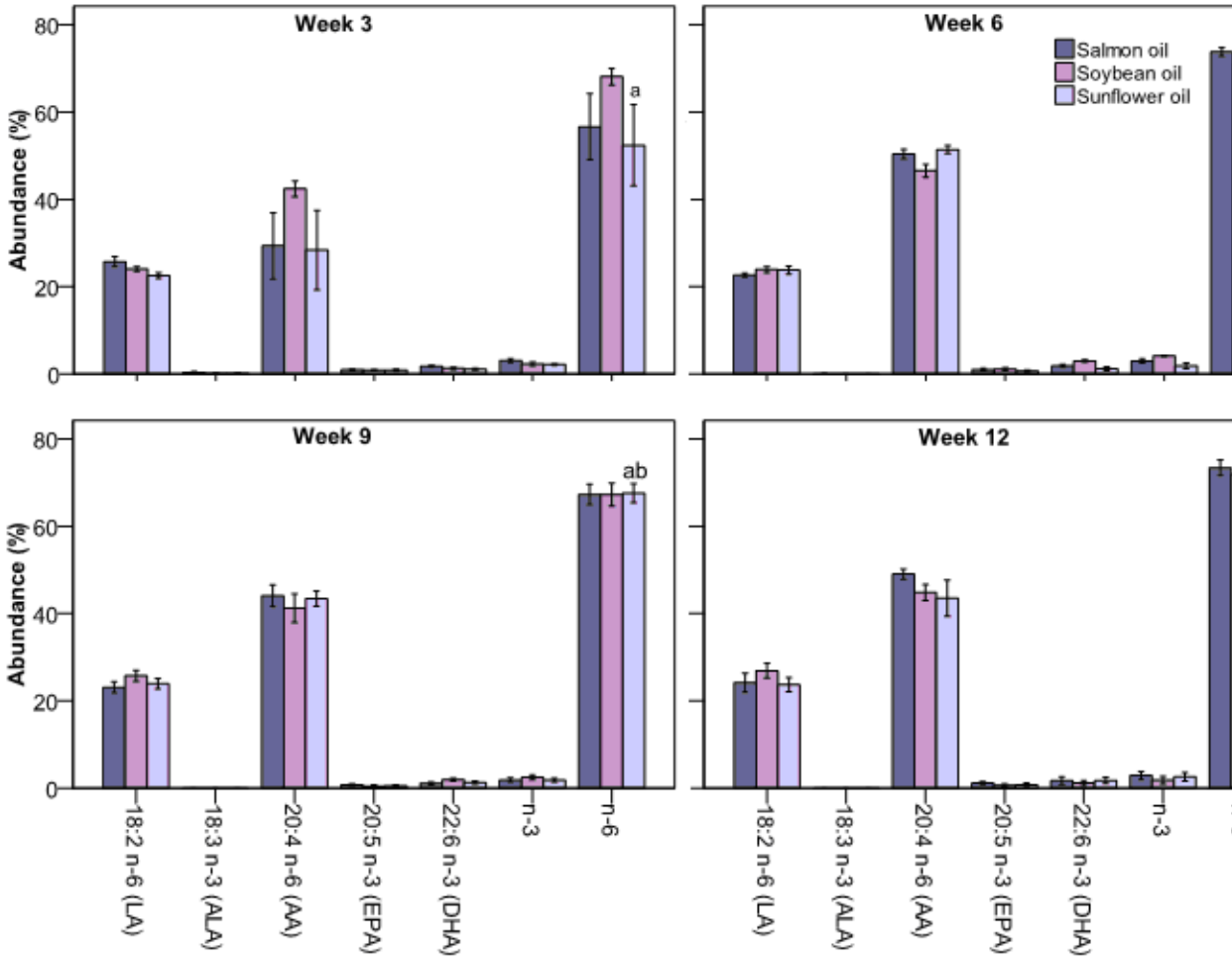

Figure 4-9: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents in the CE lipid fraction of offspring plasma. Values
differences in fatty acid abundance between different time points within a dietary group ($p<0.05$).

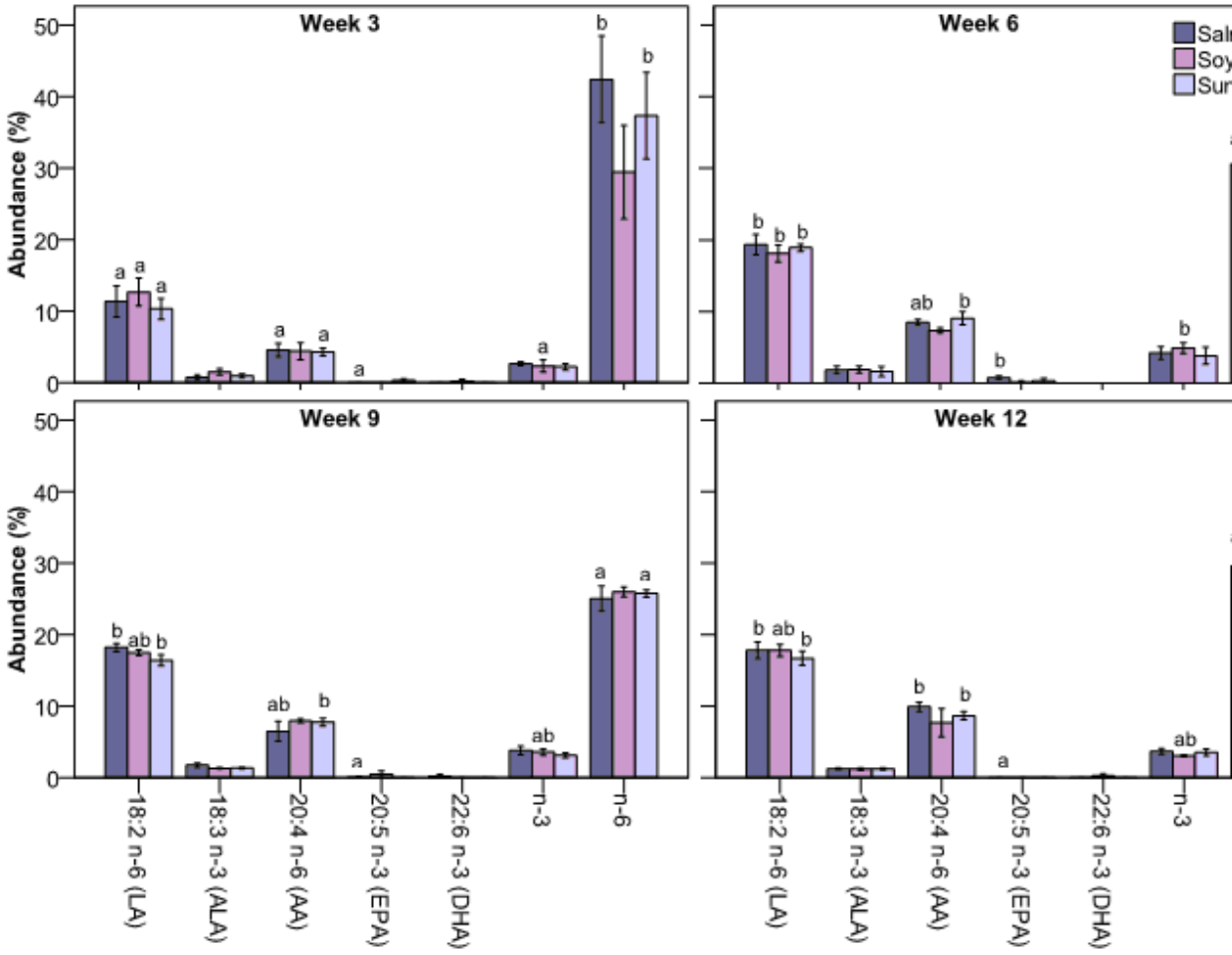

Figure 4-10: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents in the NEFA lipid fraction of offspring plasma. Value differences in fatty acid abundance between different time points within a dietary group (p<0.05).

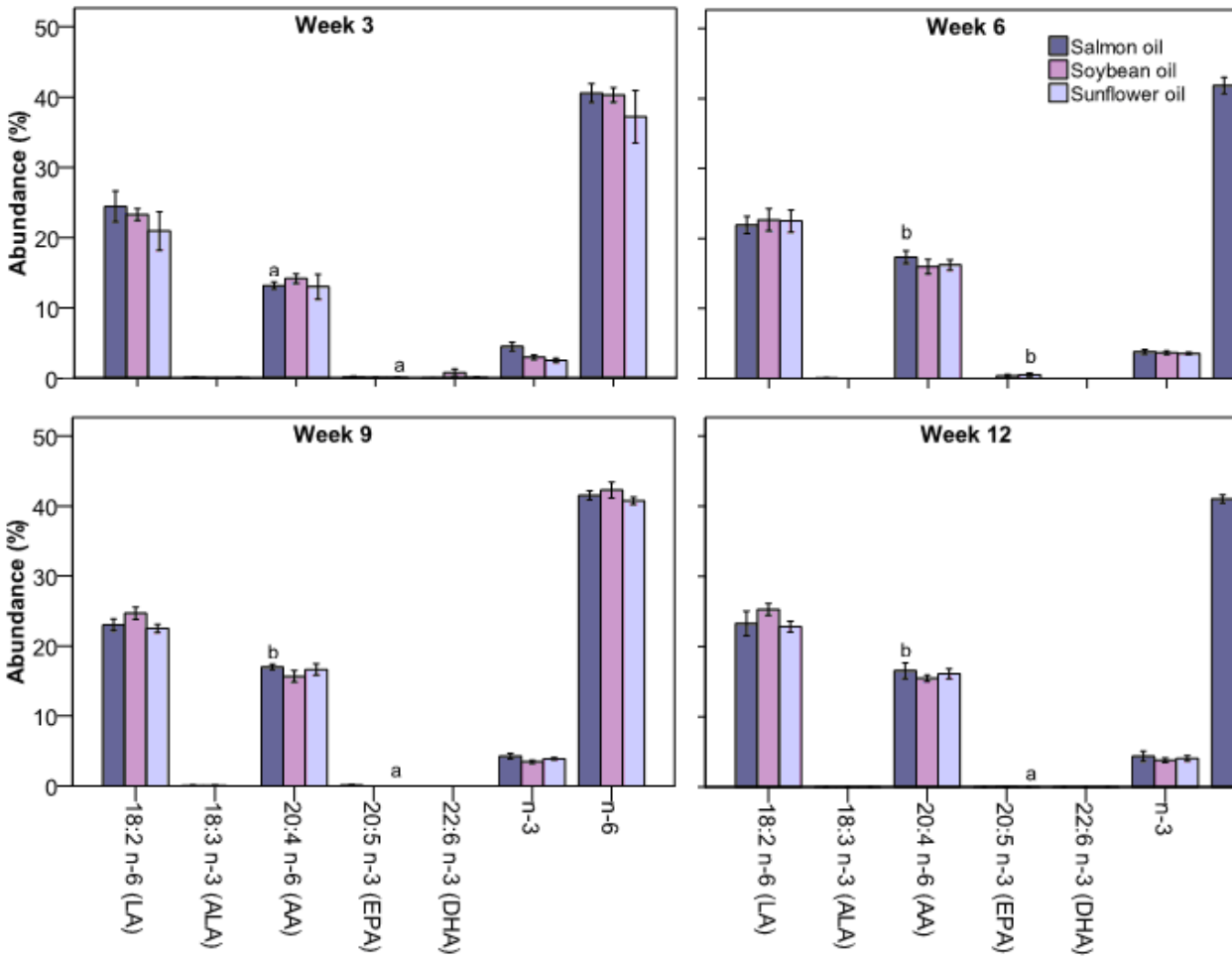

Figure 4-11: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents in the PC lipid fraction of offspring plasma. Values differences in fatty acid abundance between different time points within a dietary group ($p<0.05$).

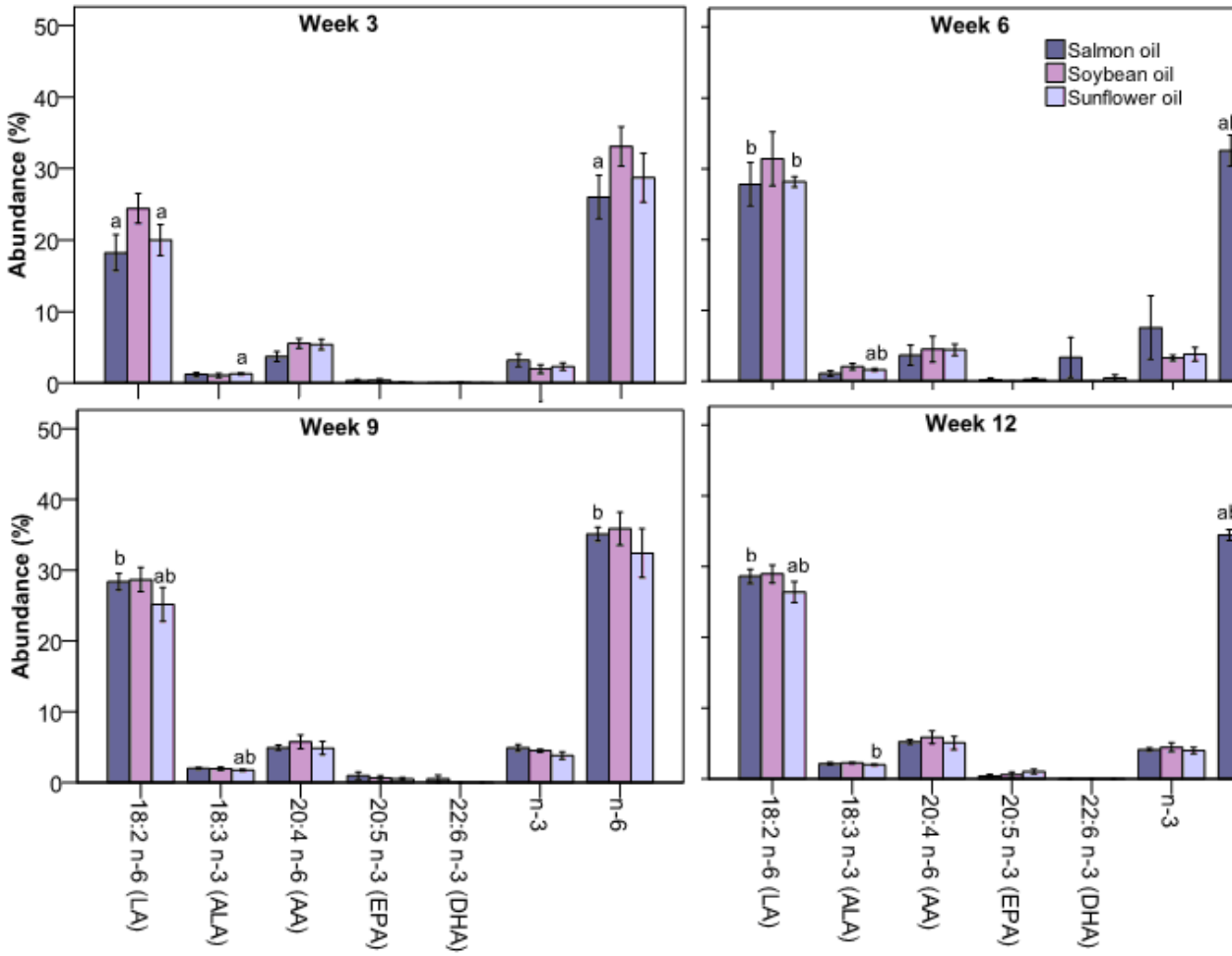

Figure 4-12: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents in the PE lipid fraction of offspring plasma. Values differences in fatty acid abundance between different time points within a dietary group (p<0.05).

4.3.7. Lymphoid Organ Cell Numbers in Offspring of Rats fed the Different Diets

Cell counts of spleen, thymus and bone marrow are shown in Tables 1-9, 1-10 and 1-11, respectively. There was no effect of time or diet on the number of spleen cells per mg of spleen tissue (Table 4-7). Absolute numbers of cells in the spleen were lower at week 3 than weeks 6, 9 and 12 across all dietary groups.

Table 4-7: Cell counts in spleen. Results are shown as mean ± SEM.

	Week		Salmon oil	Soybean oil	Sunflower oil
Number of cells per mg of spleen (x 10^4)	3	n	11	8	11
		Mean ± SEM	2.91 ± 0.46	3.57 ± 0.73	4.03 ± 0.59
	6	n	9	6	8
		Mean ± SEM	6.08 ± 1.06	3.57 ± 0.54	5.28 ± 0.57
	9	n	10	6	8
		Mean ± SEM	4.98 ± 0.62	7.06 ± 2.03	5.45 ± 1.01
	12	n	7	7	8
		Mean ± SEM	2.31 ± 0.76	4.16 ± 1.29	5.05 ± 1.97
Absolute number of cells per spleen (x 10^6)	3	n	11	8	11
		Mean ± SEM	12.03 ± 2.47	15.37 ± 4.53	14.94 ± 2.80
	6	n	9	6	8
		Mean ± SEM	60.54 ± 12.04	30.53 ± 5.10	41.69 ± 6.68
	9	n	10	6	8
		Mean ± SEM	55.80 ± 8.09	73.19 ± 22.39	57.37 ± 9.64
	12	n	5	7	8
		Mean ± SEM	40.32 ± 10.89	51.97 ± 19.17	59.66 ± 23.52

Cell counts in the thymus were made only on a sample of the tissue (as some thymus was required for histological studies by another research group). Therefore it was not possible to show data as total number of cells in the thymus; data are shown as number of cells per mg of tissue. No effect of maternal diet on thymus cell numbers was found (Table 4-8). In the salmon oil group, cell counts were higher at week 6 than at other time points. In the soybean oil and sunflower oil groups, cell counts were lowest at week 12 than at any other time point.

Table 4-8: Cell counts in thymus. Results are shown as mean ± SEM.

Week			Salmon oil	Soybean oil	Sunflower oil
Cell number per mg of thymus (x 10^4)	3	n	11	7	11
		Mean ± SEM	16.13 ± 7.73	15.22 ± 3.40	20.11 ± 4.20
	6	n	9	6	9
		Mean ± SEM	42.65 ± 8.76	29.51 ± 10.63	32.39 ± 9.02
	9	n	10	6	8
		Mean ± SEM	10.07 ± 4.78	25.51 ± 13.47	25.31 ± 7.63
	12	n	7	7	8
		Mean ± SEM	16.21 ± 8.32	5.42 ± 1.84	8.12 ± 2.11

It was not possible to record weight of bone marrow, and hence comparisons are made using the total number of cells counted. There was no effect of maternal diet on absolute cell counts of bone marrow (Table 4-9). There was no change in cell counts in the salmon oil or soybean oil groups over time. Cell counts in the sunflower oil group were higher at week 6 than at weeks 3 and 12.

Table 4-9: Absolute cell counts in marrow of tibia and femurs. Results are shown as mean ± SEM.

Week			Salmon oil	Soybean oil	Sunflower oil
Absolute number of cells in bone marrow (x 10^6)	3	n	11	8	11
		Mean ± SEM	11.87 ± 2.34	15.69 ± 2.51	10.13 ± 1.52
	6	n	9	6	7
		Mean ± SEM	19.97 ± 1.71	12.99 ± 1.57	25.28 ± 4.83
	9	n	10	5	8
		Mean ± SEM	15.09 ± 1.39	16.41 ± 2.16	17.56 ± 2.09
	12	n	7	8	9
		Mean ± SEM	17.08 ± 4.77	15.22 ± 2.54	9.88 ± 1.95

4.3.8. Immune Cell subsets in the Thymus, Spleen, Blood and Bone Marrow of Offspring fed the Different Diets

Cell types and numbers are shown below. Percentage abundance of subsets of bone marrow lymphocytes are shown in Table 4-10. There was no effect of maternal diet on abundance of cell subsets, but there were changes with time.

Table 4-10: Subsets and abundance of bone marrow lymphocytes. Results are shown as mean ± SEM. Abundance is shown as % of lymphocytes. Values with different letters denote differences in cell abundance in dietary groups between time points ($p<0.05$).

	Week		Salmon oil	Soybean oil	Sunflower oil
CD3+CD8+	3	n	7	7	7
		Mean ± SEM	1.79 ± 0.30[a]	1.80 ± 0.39[a]	1.36 ± 0.17[a]
	6	n	8	6	6
		Mean ± SEM	1.92 ± 0.26[a]	2.36 ± 0.44[ab]	1.83 ± 0.45[a]
	9	n	10	5	7
		Mean ± SEM	2.15 ± 0.45[a]	2.45 ± 0.42[ab]	1.93 ± 0.27[a]
	12	n	6	8	8
		Mean ± SEM	4.25 ± 0.39[b]	3.88 ± 0.38[b]	3.76 ± 0.40[b]
CD3+CD4+	3	n	7	7	7
		Mean ± SEM	1.41 ± 0.28	1.53 ± 0.22	1.14 ± 0.19
	6	n	8	6	6
		Mean ± SEM	2.02 ± 0.28	1.88 ± 0.40	1.87 ± 0.24
	9	n	10	4	7
		Mean ± SEM	1.29 ± 0.24	1.89 ± 0.35	1.31 ± 0.26
	12	n	6	8	8
		Mean ± SEM	2.03 ± 0.21	1.75 ± 0.16	2.02 ± 0.37
CD161+	3	n	7	7	7
		Mean ± SEM	5.55 ± 0.85[a]	5.77 ± 0.92[a]	6.04 ± 1.04[a]
	6	n	8	6	6
		Mean ± SEM	6.16 ± 1.00[a]	8.04 ± 1.41[a]	6.16 ± 1.11[a]
	9	n	8	5	7
		Mean ± SEM	19.17 ± 1.57[b]	13.24 ± 3.85[a]	12.49 ± 1.13[a]
	12	n	6	7	8
		Mean ± SEM	22.31 ± 2.13[b]	22.29 ± 2.68[b]	21.64 ± 3.04[b]
CD3-CD45RA+	3	n	7	7	7
		Mean ± SEM	36.31 ± 1.80[c]	29.11 ± 1.60	30.20 ± 2.95
	6	n	8	6	6
		Mean ± SEM	34.22 ± 2.32[bc]	34.98 ± 3.17	35.52 ± 2.76
	9	n	10	5	7
		Mean ± SEM	29.20 ± 3.41[ab]	24.37 ± 6.99	29.07 ± 5.32
	12	n	6	8	8
		Mean ± SEM	23.71 ± 1.93[a]	20.70 ± 3.80	21.10 ± 2.98
Unidentified	3	n	7	7	7
		Mean ± SEM	54.94 ± 2.41	61.80 ± 1.43	61.26 ± 2.93
	6	n	8	6	6
		Mean ± SEM	55.68 ± 2.20	52.74 ± 2.36	54.62 ± 3.24
	9	n	10	5	7
		Mean ± SEM	52.02 ± 3.21	58.43 ± 8.85	55.20 ± 4.76
	12	n	6	8	8
		Mean ± SEM	47.70 ± 3.52	54.16 ± 4.16	51.48 ± 1.74

Over time, the percentage of CD3+CD8+ cells (cytotoxic T cells) increased. In the salmon oil and sunflower oil groups CD3+CD8+ cells were more abundant at week 12 than at weeks 3, 6 and 9. In the soybean oil group percentage of CD3+CD8+ cells was higher at week 12 than at week 3. There was no change in percentage of CD3+CD4+ cells (T helper cells) across dietary groups. In the soybean oil and sunflower oil groups, percentage of CD161+ cells (NK cells) was higher at week 12 than at weeks 3, 6 and 9. In the salmon oil

group, CD161+ cell abundance was higher at weeks 9 and 12 than at weeks 3 and 6. There was no effect of time in CD3-CD45RA+ abundance in the soybean oil group, but there was a gradual reduction seen in the salmon oil and sunflower oil groups. The “unidentified” section is the percentage of cells that are unaccounted for by the markers used. There was no effect of time or diet on level of unidentified cells.

Percentage of subsets of splenic lymphocytes are shown in Table 4-11.

Table 4-11: Abundance of subsets of splenic lymphocytes. Results are shown as mean ± SEM. Abundance is shown as % of splenocytes. Values with different letters denote differences in cell abundance in dietary groups between time points (p<0.05).

	Week		Salmon oil	Soybean oil	Sunflower oil
CD3+CD8+	3	n	7	7	7
		Mean ± SEM	6.81 ± 0.40[a]	7.94 ± 1.39[a]	7.27 ± 0.43[a]
	6	n	8	6	7
		Mean ± SEM	13.01 ± 1.10[ab]	17.22 ± 2.55[ab]	13.73 ± 1.78[ab]
	9	n	8	4	6
		Mean ± SEM	17.75 ± 1.94[b]	19.29 ± 1.50[b]	14.31 ± 1.60[b]
	12	n	7	8	8
		Mean ± SEM	17.26 ± 2.36[b]	20.76 ± 2.86[b]	20.61 ± 2.91[b]
CD3+CD4+	3	n	7	7	7
		Mean ± SEM	13.08 ± 1.43[a]	12.46 ± 1.64[a]	12.86 ± 1.15[a]
	6	n	8	6	7
		Mean ± SEM	22.19 ± 1.66[b]	23.60 ± 2.66[b]	23.56 ± 1.90[b]
	9	n	8	4	7
		Mean ± SEM	25.14 ± 2.45[b]	28.73 ± 3.07[b]	30.23 ± 3.04[b]
	12	n	7	8	8
		Mean ± SEM	28.15 ± 2.54[b]	29.84 ± 2.88[b]	27.77 ± 1.91[b]
CD161+	3	n	7	7	7
		Mean ± SEM	1.63 ± 0.22	1.57 ± 0.14	1.32 ± 0.26
	6	n	8	6	7
		Mean ± SEM	1.52 ± 0.28	1.50 ± 0.23	1.40 ± 0.27
	9	n	8	4	7
		Mean ± SEM	2.85 ± 0.60	1.94 ± 0.36	1.84 ± 0.22
	12	n	7	8	8
		Mean ± SEM	1.67 ± 0.33	1.84 ± 0.35	1.36 ± 0.19
CD3-CD45RA+	3	n	7	7	7
		Mean ± SEM	56.72 ± 4.16[b]	44.70 ± 2.39	40.23 ± 7.61
	6	n	8	6	7
		Mean ± SEM	46.12 ± 2.27[ab]	42.59 ± 1.83	39.69 ± 3.89
	9	n	8	4	7
		Mean ± SEM	37.31 ± 5.28[a]	39.12 ± 0.68	32.45 ± 6.68
	12	n	7	8	8
		Mean ± SEM	40.50 ± 3.25[a]	41.10 ± 2.54	34.65 ± 5.15
CD163+	3	n	7	7	7
		Mean ± SEM	4.84 ± 1.37[a]	4.53 ± 1.41[a]	3.14 ± 0.85[a]
	6	n	8	6	7
		Mean ± SEM	5.03 ± 0.55[a]	9.27 ± 2.27[ab]	5.21 ± 0.88[a]
	9	n	8	4	7
		Mean ± SEM	13.79 ± 1.53[b]	14.55 ± 2.69[b]	12.54 ± 1.25[b]
	12	n	7	8	8
		Mean ± SEM	12.48 ± 1.60[b]	14.75 ± 2.27[b]	13.32 ± 2.35[b]
Unidentified	3	n	7	7	7
		Mean ± SEM	16.92 ± 2.22[b]	28.81 ± 2.62[b]	35.18 ± 8.63[b]
	6	n	8	6	7
		Mean ± SEM	12.87 ± 3.54[ab]	8.08 ± 2.96[a]	16.41 ± 4.31[ab]
	9	n	8	4	7
		Mean ± SEM	5.97 ± 2.20[ab]	2.71 ± 2.22[a]	10.70 ± 4.83[a]
	12	n	7	8	8
		Mean ± SEM	3.85 ± 2.98[a]	0.45 ± 0.45[a]	7.36 ± 3.47[a]

There was no effect of diet on abundance of any of the markers measured, although there was a trend towards diet affecting B-cell abundance ($p = 0.058$). In the salmon oil and soybean oil groups, percentage of CD3+CD8+ cells was lower at week 3 than at weeks 9 and 12. In the sunflower oil group CD3+CD8+ cells were more abundant at week 12 than week 3. In all groups, CD3+CD4+ cells were less abundant at week 3 than at weeks 6, 9 and 12. There was no effect of time on CD161+ cell abundance. The sunflower oil and soybean oil groups showed no change in CD3-CD45RA+ abundance with time, but in the salmon oil group, CD3-CD45RA+ were in greater abundance at week 3 than at weeks 9 and 12. CD163+ cell expression in the salmon oil and sunflower oil groups were lower at weeks 3 and 6 than weeks 9 and 12. In the soybean oil group, CD163+ expression was in lower abundance at week 3 than weeks 9 and 12. The percentage of cells that were unidentified by the markers used decreased with time.

Percentage abundance of thymocytes is shown in Table 4-12.

Table 4-12: Abundance of subsets of thymic cells. Results are shown as mean ± SEM. Abundance is shown as % thymic cells. Values with different letters denote differences in cell abundance in dietary groups between time points ($p<0.05$).

	Week		Salmon oil	Soybean oil	Sunflower oil
CD3+CD8+	3	n	6	6	7
		Mean ± SEM	9.88 ± 1.81[a]	12.62 ± 2.46	11.99 ± 1.03
	6	n	9	6	8
		Mean ± SEM	12.76 ± 1.49[ab]	13.27 ± 2.03	14.15 ± 1.65
	9	n	8	5	6
		Mean ± SEM	16.84 ± 2.06[ab]	18.97 ± 3.97	16.90 ± 2.52
	12	n	7	8	9
		Mean ± SEM	19.26 ± 3.35[b]	21.10 ± 3.09	17.78 ± 1.99
CD3+CD4+	3	n	7	6	7
		Mean ± SEM	20.47 ± 2.69	19.52 ± 3.51	21.09 ± 2.31
	6	n	8	6	8
		Mean ± SEM	20.44 ± 1.94	21.42 ± 3.78	23.46 ± 3.64
	9	n	8	6	6
		Mean ± SEM	30.48 ± 3.02	24.83 ± 3.57	25.83 ± 2.86
	12	n	7	8	9
		Mean ± SEM	28.00 ± 3.76	28.99 ± 2.03	30.63 ± 2.33
CD161+	3	n	7	6	6
		Mean ± SEM	0.39 ± 0.05[a]	0.47 ± 0.18	0.50 ± 0.17[a]
	6	n	9	5	8
		Mean ± SEM	0.32 ± 0.06[a]	0.46 ± 0.13	0.33 ± 0.02[a]
	9	n	8	6	6
		Mean ± SEM	1.12 ± 0.19[b]	0.56 ± 0.17	0.54 ± 0.14[a]
	12	n	7	6	9
		Mean ± SEM	1.17 ± 0.28[b]	0.94 ± 0.24	1.24 ± 0.20[b]
CD3-CD45RA+	3	n	7	6	6
		Mean ± SEM	0.54 ± 0.11[a]	0.32 ± 0.05[a]	0.68 ± 0.25[a]
	6	n	9	6	8
		Mean ± SEM	0.46 ± 0.12[a]	0.45 ± 0.04[a]	0.47 ± 0.10[a]
	9	n	8	6	6
		Mean ± SEM	1.81 ± 0.31[b]	1.07 ± 0.18[b]	1.27 ± 0.38[ab]
	12	n	7	8	9
		Mean ± SEM	1.73 ± 0.31[b]	2.03 ± 0.08[c]	2.31 ± 0.35[b]
Unidentified	3	n	7	6	7
		Mean ± SEM	70.13 ± 3.78[b]	67.07 ± 6.09	65.90 ± 2.91[b]
	6	n	9	6	8
		Mean ± SEM	68.29 ± 4.13[ab]	64.48 ± 5.67	61.59 ± 5.02[ab]
	9	n	8	6	6
		Mean ± SEM	49.76 ± 5.25[a]	57.74 ± 7.97	55.46 ± 5.10[ab]
	12	n	7	8	9
		Mean ± SEM	49.84 ± 7.44[ab]	47.18 ±4.47	48.04 ± 4.24[a]

There was no effect of diet on any of parameters measured. There was no change in CD3+CD8+ abundance over time in the soybean oil and sunflower oil groups, but the salmon oil group showed lower abundance at week 3 than week 12. There was no change in CD3+CD4+ expression across the time points measured. CD161+ cells were lower at week 3 and 6 compared to 9 and 12 in the salmon oil group, whilst there was no change in the soybean oil group. In the sunflower oil group, CD161+ cells were higher at week 12

than at weeks 3, 6 and 9. CD3-CD45RA+ in the salmon oil group were significantly lower at weeks 3 and 6 than at weeks 9 and 12. In the soybean oil group, there was no difference between CD3-CD45RA+ cell abundance at weeks 3 and 6, but there was an increase at week 9 and again at week 12. CD3-CD45RA+ expression at week 12 in the sunflower oil group were higher than at weeks 3 and 6. There was no effect of time on percentage of cells that could not be identified in the soybean oil group, but there was a significant reduction between weeks 3 and 12 in the other two dietary groups.

Effect of diet in pregnancy on offspring immune cells of whole blood is shown in Table 4-13.

Table 4-13: Abundance of immune cells in whole blood. Results are shown as mean ± SEM. Abundance is shown as % of lymphocytes Values with different letters denote differences in cell abundance in dietary groups between time points (p<0.05).

			Salmon oil	Soybean oil	Sunflower oil
CD3+CD8+	3	n	6	7	6
		Mean ± SEM	8.61 ± 1.23	7.96 ± 2.02	3.31 ± 0.82
	6	n	8	5	6
		Mean ± SEM	9.44 ± 2.85	5.99 ± 1.70	9.92 ± 0.61
	9	n	9	8	12
		Mean ± SEM	4.31 ± 0.80	4.69 ± 1.12	8.32 ± 1.91
	12	n	6	5	6
		Mean ± SEM	6.63 ± 1.53	5.99 ± 1.92	5.70 ± 1.59
CD3+CD4+	3	n	6	7	6
		Mean ± SEM	13.18 ± 2.96[a]	14.08 ± 1.70	10.77 ± 3.32
	6	n	8	5	7
		Mean ± SEM	11.39 ± 1.85[b]	11.86 ± 3.74	20.30 ± 2.02
	9	n	9	7	12
		Mean ± SEM	6.88 ± 1.47[ab]	8.69 ± 1.90	13.86 ± 1.77
	12	n	6	5	6
		Mean ± SEM	10.44 ± 1.68[b]	10.34 ± 3.71	8.17 ± 2.00
CD161+	3	n	6	7	6
		Mean ± SEM	5.18 ± 2.16[a]	6.79 ± 2.55	5.54 ± 2.10
	6	n	7	5	7
		Mean ± SEM	6.07 ± 2.70[a]	9.08 ± 2.60	7.61 ± 1.98
	9	n	9	8	11
		Mean ± SEM	10.48 ± 1.54[ab]	9.99 ± 2.12	13.04 ± 1.99
	12	n	6	5	6
		Mean ± SEM	15.51 ± 0.94[b]	10.30 ± 3.26	9.91 ± 2.06
CD3-CD45RA+	3	n	5	7	6
		Mean ± SEM	30.01 ± 7.06	20.90 ± 1.64	17.03 ± 3.44
	6	n	8	5	7
		Mean ± SEM	18.10 ± 3.81	16.84 ± 3.92	22.96 ± 2.89
	9	n	9	8	12
		Mean ± SEM	16.88 ± 2.51	21.83 ±1.92	23.40 ± 2.00
	12	n	6	5	6
		Mean ± SEM	22.95 ± 2.81	18.87 ± 3.71	19.51 ± 2.24
Unidentified	3	n	5	7	6
		Mean ± SEM	42.33 ± 7.83	52.99 ± 5.98	63.35 ± 4.15[c]
	6	n	7	5	6
		Mean ± SEM	53.76 ± 6.17	56.23 ±8.75	38.94 ± 3.20[a]
	9	n	9	7	11
		Mean ± SEM	61.44 ± 4.38	55.89 ± 2.38	40.43 ± 3.60[ab]
	12	n	6	5	6
		Mean ± SEM	44.48 ± 4.44	54.50 ± 5.52	56.71 ± 5.49[bc]

There was no effect of diet on the expression of cells measured in blood. In addition, there was no effect of time on abundance of CD3+CD8+ or CD3-CD45RA+ expression. There was no effect of time on the abundance of CD3+CD4+ in the salmon oil or soybean oil groups. CD3+CD4+ expression was higher at week 6 than at weeks 3 and 12 in the sunflower oil group. There was no effect of time on the abundance of CD161+

cells in the soybean or sunflower oil groups. Expression of CD161+ in the salmon oil group was higher at week 6 than at weeks 3 and 12. There was no effect of time on the abundance of unidentified cells in the salmon and soybean oil groups. In the sunflower oil group, unidentified cells were higher at week 3 than at weeks 6 and 9, and higher at week 12 than at week 6.

4.4. Discussion

4.4.1. Summary of findings

This study aimed to determine whether changes in fatty acid composition of the maternal diet may cause changes in the structure of foetal and / or maternal immune cells, which in turn may alter the immune system of offspring, following on from previous research, which found some effect in foetal rats at around the time of birth [133, 217]. The study also aimed to determine whether these changes were maintained in later life, at weaning and then beyond weaning when offspring are no longer directly affected by maternal diet. The study showed that altering maternal dietary fatty acid intake in pregnancy did not affect birthing outcomes, tissue weights, abundance of lymphocytes or subsets of immune cells in spleen, thymus, bone marrow or whole blood in the offspring at age 3, 6, 9 or 12 weeks. The fatty acid profile of maternal plasma at the end of weaning differed between dietary groups, and reflected fatty acid intake in pregnancy. There was no effect of diet on fatty acid profile of offspring, although there were changes with time.

4.4.2. Gestational Data

In addition to their roles in modulation of the immune system, the importance of fatty acids in pregnancy has long been recognised [222]. Research has suggested that heightened *n*-3 PUFA intake in pregnancy may increase gestation length (reviewed by Abayasekara and Wathes [223] and Jensen [224]). This could occur as a result of decreasing PGE_2 and increasing PGE_3 level, the latter being thought to be less potent at inducing contractions than PGE_2. However, the current study found no difference in terms of gestation length according to diet. This may be an artefact since births were recorded either early in the morning or at the end of the day; any births happening after 5 pm would therefore be recorded as having occurred the next day.

Although twelve dams had originally been assigned to each dietary group, only nine (soybean oil group) or eleven (salmon oil and sunflower oil groups) animals had successful birthing outcomes (i.e. delivered and reared offspring). It is useful to note that the dietary groups had similar success rates of delivery, as failure of successful delivery is therefore unlikely to be the result of a particular fatty acid(s). However, it cannot be ruled out that it could be the result of a high fat diet, or a diet rich in PUFAs in pregnancy without the creation of an additional group(s) that would be fed a diet low in PUFAs (such as chow or one that uses beef tallow as a fat source) during gestation.

4.4.3. Body and Tissue Weights of Offspring

Spleen and thymus weights were highly correlated to body weight in both male and female offspring. However, there was no difference between body, spleen or thymus weight as a result of diet in pregnancy in either males or females. Korotkova *et al.* [152] found a gender-related long-term effect on body weights of offspring of feeding PUFAs in pregnancy. Rats were fed one of three diets; an *n-3* diet (linseed oil), an *n-3* / *n-6* diet (soybean oil) or an *n-6* diet (sunflower oil). Males in the soybean oil group were heavier than those in the linseed oil or sunflower oil groups. Females in the soybean oil group were heavier than those from the linseed oil group, which in turn were heavier than those in the sunflower oil group. However, this study used linseed oil as the *n-3* source, which may induce a more obvious gender effect than using salmon oil (discussed further in 4.4.6).

There has been much debate on the effect of *n-3* PUFAs on body weight at birth (reviewed by Jensen [224] and Szajewska *et al.* [225]).Whilst it would have been interesting to determine the birth weight of the litter in the current trial, this was not carried out since handling in the immediate post-partum period can cause infanticide [3].

4.4.4. Fatty Acid Content of Maternal Plasma and Tissues, and Offspring Plasma

The fatty acid profile of maternal plasma at the end of weaning to the most part reflected fatty acid intake in pregnancy, although changes were not as extreme as might be expected as animals had been receiving chow for three weeks prior to blood and tissue collection, and fatty acid profile of plasma reflects a more recent intake of fatty acid than fatty acid profile of other tissues or blood components, such as erythrocytes.

In the CE fraction of maternal plasma, 18:2 *n-6* content was higher in the salmon oil group than the sunflower oil group. This is not as was expected, as the 18:2 *n-6* content of diet was lower in the salmon oil diet than the sunflower oil diet. However, the chow diet (which dams received following birth) contained a similar percentage of 18:2 *n-6* as the soybean and sunflower oil diets, which may explain the higher content in plasma of 18:2 *n-6* in salmon oil group compared to sunflower oil group. Alternatively, 18:2 *n-6* may have been sourced from the dam's stores, laid down prior to pregnancy, in order to meet demands of lactation. 20:5 *n-3*, total *n-3* contents and ratio of *n-3* to *n-6* PUFAs were highest in the plasma of the salmon oil dams.

There was no difference in fatty acid profile in the NEFA fraction between dietary groups. This is as would be expected, since the NEFA fraction has a short half life in the

blood, and partly represents recently absorbed dietary fatty acids [18]; dams from all groups had been receiving chow since parturition, three weeks previously.

Trans 18:1 *n-9* in the PC fraction of maternal plasma were highest, and 18:3 *n-6* and 20:3 *n-6* levels were lowest in the in the soybean oil group compared to other dietary groups. *Trans* 18:1 *n-9* content of diets was not determined. 20:5 *n-3* levels were higher in the salmon oil group compared to the soybean oil group. That there was no significant difference between 20:5 *n-3* plasma levels between the salmon oil and sunflower oil groups was not as expected. There may have been an increased demand of 20:5 *n-3* in dams receiving the sunflower oil diet because of the lower intake of 20:5 *n-3* in pregnancy. Whilst the soybean oil group had a low intake of 20:5 *n-3* in pregnancy, they had a higher intake of its shorter chain precursor, and as such may not have shown an increased demand and subsequent circulation of 20:5 *n-3* that dams in the sunflower oil group did.

Trans 18:1 *n-9* levels in the TAG fraction were higher in the soybean oil group than the sunflower oil group. In the fatty acid analysis of diets, the soybean oil group contained higher levels of *trans* 18:1 *n-9* levels than the sunflower oil group, but lower levels than the salmon oil group. *Trans* 18:1 *n-9* content of chow was not determined, but the fat source of chow is soybean oil, and therefore, it might be reasoned that chow would have a similar percentage abundance of this fatty acid (albeit a lower absolute amount). *Trans* 18:1 *n-9* was higher in the salmon oil diet compared to the soybean oil diet, this difference was not reflected in the fatty acid profile of plasma, and may be due to the chow received during lactation. Whilst, the soybean oil group continued receiving higher levels of *trans* 18:1 *n-9* during lactation, and plasma levels of *trans* 18:1 *n-9* in the sunflower oil group increased, they did not do so to the level of that in the soybean oil group.

To date, trials have not examined the effect of fatty acid intake in pregnancy on fatty acid profile of plasma of dams at the end of lactation. As such, it is important to also examine the effect on fatty acid profile of tissues of dams. As with the maternal plasma samples, tissue samples were taken three weeks after parturition, and changes that may have been seen if samples had been taken earlier, may not be seen. It was expected that tissues in which fatty acids were stored may be less susceptible to the change in diet and effect of time. In the liver, which acts as a short-term storage area for fatty acids, there were no differences in fatty acid content between groups except for the PC and PE fractions. In the thymus and spleen differences in fatty acid content were observed in both these fractions and also the NEFA fraction. In all three tissues, fatty acid content reflected differences in diet during pregnancy.

Despite the differences observed in maternal tissue and plasma, there was no effect of diet in pregnancy on offspring fatty acid profile in plasma, although there was an effect of time. This is likely to be due to the fact that plasma reflects the more recent dietary fatty acid intake and changes may therefore have been detected in other samples, such as erythrocytes, which reflect the dietary fatty acid intake less recently, that were not observed here. The effect of time may be due to the fact that from birth, animals received milk from a mother who was consuming chow, or they were themselves receiving chow, which they were weaned onto at 3 weeks of age. Fatty acid profile of plasma may have "normalised" over time to reflect the effect of a longer intake of chow. Whilst differences between dietary groups were not significant, there may have been enough of a difference to account for there being no apparent difference between groups at a later time point, but there being an effect of time.

4.4.5. Cell Counts and Subsets in the Thymus, Spleen, Blood and Bone Marrow

In agreement with the findings reported in Chapter 2 and Chapter 3, there was no effect of fatty acid intake in pregnancy on the abundance of lymphocytes, NK cell, T_H cells, CTLs, or macrophages. Research has suggested that balance of *n-3* and *n-6* PUFAs may affect balance of T_H1 and T_H2 cells (1.4). It had originally been intended to examine the balance of these cells by analysis of IFN-γ (a characteristic cytokine of T_H1 cells) and IL-4 (a characteristic cytokine of T_H2 cells) abundance following cell culture. However, there were insufficient cells to allow this.

4.4.6. Limitations

There are a few limitations of this trial; areas in which further investigation could be carried out. For example, it would be important to know whether the fatty acid manipulation of the gestational diet affected the fatty acids deposited in lymphoid organs or in the blood of offspring. However, there were insufficient cells available to allow this. It would also be prudent to determine whether changes can be determined in the fatty acid deposition in dams at the end of pregnancy. This was not carried out in this trial, however, as the dams were required for rearing offspring, which prohibited tissue collection. Samples were collected from dams at sacrifice when offspring were weaned, and remain to be analysed.

It is possible to collect blood by tail vein sampling of dams, although this could further reduce likelihood of successful birthing outcomes and was outside the remit of the project and personal licenses. In addition it would have been beneficial to collect and analyse the fatty acid composition of breast milk, to determine whether the experimental diets are having an effect during lactation (by which time all animals were receiving chow). However, due to limitations of Home Office licenses, these investigations were not carried out in this trial.

Several studies have suggested an effect of gender on the metabolism and usage of fatty acids, in particular *n-3* PUFAs [133, 152, 201, 218, 226-228]. As such, there may be a gender-effect of *n*-3 PUFAs in pregnancy due to the increased metabolism of ALA in females compared to males [201, 227], that would not have been realised in this study due to the fact that each offspring sample contained tissue from one male and one female pup. However, the salmon diets are naturally high in long-chain *n-3* PUFAs, so any effect of gender conversion on ALA may be less important than it would be in, for example, groups fed diets high in the shorter *n-3* PUFAs, such as in linseed oil-based feed. This may explain the reason that a difference in body weight was observed in the study by Korotkova *et al.* [152], who gave a linseed oil-based diet to pregnant rats, whilst a difference was not observed in the current study, which used a salmon oil-based diet.

4.4.7. Conclusions

This study hypothesised that changes in fatty acid intake in pregnancy would affect the fatty acid composition of maternal plasma and tissues (at the end of lactation) and of offspring plasma at weaning and beyond, and would affect the abundance of immune cells in the lympoid tissues of the offspring. The fatty acid composition of the rat diet during pregnancy did affect the fatty acid composition of maternal plasma, liver, spleen and thymus at the end of lactation. However there was no effect on offspring fatty acid composition at weaning and beyond or on lymphoid organ immune cell subsets. Thus it is concluded that the effects of maternal fatty acids on late foetal fatty acid composition and thymus lymphocyte sub-populations [133, 217, 218]are not retained at weaning or beyond if the dams are transferred to a chow diet after giving birth. Thus, these parameters appear not to be programmed by maternal fatty acid exposures. However, *n-3* and *n-6* PUFA balance in pregnancy may affect immune markers not measured in this study, such as the balance of T_H1 and T_H2 cells, and subsequently immune reactions. In addition, the functional consequences of any changes (that were not measurable in this trial) remain to be determined. It may be of particular importance to examine these functional consequences

in vivo, as the immune system is a complex of interacting factors and not the result of the action of one singular type of cell(s). For example, dietary intake in pregnancy may affect the development of allergy or immune response. These ideas will be explored further in the following chapters.

Chapter 5. Effect of Early Exposure to Fatty Acids on Later Allergic Sensitisation Examined using a Mouse Model

5.1. Introduction

Findings from Chapter 3 suggest that allergic sensitisation in the mouse may be affected by dietary fatty acids. In addition, studies to date have examined either the effect of long chain *n-3* PUFAs in pregnancy on allergic sensitisation of offspring, or the effect of short chain PUFAs in non-pregnant beings on allergic sensitisation. Therefore it was decided to examine the effect of short chain PUFAs in pregnancy and lactation on allergic sensitisation of the offspring, to see if the immune system was affected by these fatty acids at certain periods during development. In collaboration with the University of Utrecht, an experiment was carried out to determine the effect of fatty acids given during gestation and / or lactation on development of allergic sensitisation in the offspring; mice were used. Due to the number of dietary groups that would be involved if all diets from Chapter 3were used, practical constraints limited the number of experimental diets to two; the two experimental diets were rich in either one of the essential fatty acids (EFAs) LA or ALA. The author was involved in tissue and data collection on the day of euthanisation, whilst Dr. Astrid Hogenkamp and Dr. Naomi van Vlies at the University of Utrecht carried out feeding and experiments prior to this. The author analysed all data produced.

5.1.1. EFAs and Pregnancy / Lactation

A more in-depth discussion of fatty acids (including EFAs) in pregnancy is included in 1.6. However, it is important to note that in pregnancy ALA and LA are of critical importance to foetal growth and development, because of their roles as components of membrane lipids, regulators of gene expression, and precursors of longer chain PUFAs and subsequently eicosanoids, in cellular communication, and their direct interactions with proteins [203]. As ALA and LA cannot be formed *de novo*, during gestation they must be present in the maternal diet, and be transferred across the placenta; after birth ALA and LA must be obtained from the infant diet, including breast milk during lactation [204].

The fatty acid composition of milk varies due to several factors. Human breast milk composition varies over the course of the day, during the course of suckling, over the course of lactation, and between individuals [229]. Fatty acid composition of milk is affected by maternal diet before and during lactation, by maternal metabolism, and by the extent to which the breast was emptied of milk on the previous feed [229, 230]. Emmett and Rogers [229] suggest that fatty acid composition is the most variable component of breast milk.

Fatty acids are synthesised in the mammary gland, from fatty acids sourced from recent dietary intake, from maternal adipose tissue or *de novo* synthesis or metabolism in

the maternal liver [231]. LA and ALA are only sourced from recent dietary intake or maternal adipose tissue. Breast milk provides a wide range of lipids [231] which are present as globules emulsified in the aqueous fraction of milk. Fat in breast milk is composed predominately of TAGs (98 %) [232]. Phospholipids (PLs) are thought to contribute to approximately 0.8 % of milk fat, although content determined can be affected by the method of assessment used [232]. Cholesterol accounts for approximately 0.5 % of milk fat. The remainder of lipids in milk are made up by sphingomyelin, glycosphingolipids and glucosylceramide [232]. Lipids in milk are absorbed by the infant as described in 1.3.3, with absorption of fatty acids from breast milk aided by the presence of lipases secreted in milk [21].

5.1.2. EFAs in Pregnancy and Allergy Development in Later Life

The foetal immune system starts to develop before birth, and continues postnatally, and as such, the foetus and newborn may be more sensitive to the effects of dietary fatty acids than adults. Previous research has also suggested that fish oil in pregnancy has the potential to reduce allergic sensitisation [233, 234]. However, there is a dearth of research on the effect of EFAs in pregnancy and / or lactation on the development of allergies in later life. Duchén *et al.* [235] suggested a relationship between low ALA and long chain *n-3* PUFAs content of breast milk and an increase in symptoms of allergic disease at 18 months. Research has suggested that diet in pregnancy may be similar in women to diet outside of pregnancy [236], and potentially, therefore, diet onto which the child is weaned could be similar to the mother's diet after and during pregnancy. Therefore, Duchén *et al.* may have missed a critical window of effect, or rather, encompass this critical window, but also include other periods of time at which fatty acids may not affect the immune system. Whilst Duchén *et al.* does not provide a conclusive link, or identify any temporal effects, it does suggest that there is potentially a link between EFAs and allergy development. The effect of diet in lactation also needs further exploration; it is undetermined whether fatty acids either in lactation alone, or during both pregnancy and lactation, have any effect on development of allergy in offspring.

5.1.3. Aims of the Study

This study aimed to determine the effect of diets containing differing levels of LA and ALA given during gestation and / or lactation, on the immune response in a mouse

model of allergy to the egg protein ovalbumin. Altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing them to become more or less susceptible to their allergy. In addition, mice may be more susceptible to the effects of fatty acids at certain periods (i.e. gestation and / or lactation) if a critical window is in effect.

In order to achieve this aim, three diets were fed to 8-week old mice during gestation and / or lactation. In contrast to previous experiments, experimental diets contained a synthetic blend of oils, in order to determine the effect of specific fatty acids on the immune system. The first diet was a high fat (10 %) LA-rich diet, and the second a high fat (10 %) ALA-rich diet. The third diet was chow, a lower fat (7 % diet) diet whose fat source is soybean oil (and therefore contained a balance of LA and ALA). Mice were then split into seven groups using these diets. One group received chow during both gestation and lactation (Control). The others received the LA diet in gestation and chow during lactation (LG), the LA diet during both gestation and lactation (LGL), chow during gestation and LA diet during lactation (LL), ALA during gestation and chow at lactation (AG), ALA during both gestation and lactation (AGL), or chow during gestation and ALA during lactation (AL). Following lactation, male offspring were weaned onto a diet formulated to represent a Western diet; this was rich in SFAs and MFAs ("Western" diet). This is summarised in Table 5-1.

Table 5-1: Diets used in this trial.

Group	Gestation Diet	Lactation Diet	Diet From Weaning
Control	Chow	Chow	"Western" diet
AG	ALA-rich	Chow	"Western" diet
AGL	ALA-rich	ALA-rich	"Western" diet
AL	Chow	ALA-rich	"Western" diet
LG	LA-rich	Chow	"Western" diet
LGL	LA-rich	LA-rich	"Western" diet
LL	Chow	LA-rich	"Western" diet

5.1.4. Hypothesis

This study set out to test the following hypotheses:

- Feeding the ALA-rich diet in pregnancy will reduce the severity of allergic reactions
- Feeding ALA-rich diet in pregnancy (but not lactation) will reduce the severity of allergic reactions more than feeding ALA-rich diet in lactation (but not pregnancy)

- Feeding the ALA-rich diet in both pregnancy and lactation will result in the same severity of allergic reactions as feeding the ALA-rich diet in pregnancy alone
- Feeding the LA-rich diet in pregnancy will increase the severity of allergic reactions
- Feeding the LA-rich diet in pregnancy (but not lactation) will increase the severity of allergic reactions more than feeding LA-rich diet in lactation (but not pregnancy)
- Feeding the LA-rich diet in both pregnancy and lactation will result in the same severity of allergic reactions as feeding the LA-rich diet in pregnancy alone

5.2. Materials and Methods

5.2.1. Animals, Diets and Materials

5.2.1.1. Animals

8 week old non-nulliparous BALB/C mice were obtained from Charles River (Maastricht). Following mating, dams were housed in individually in shoebox cages, and kept at 22°C ± 2°C, under a 12 hour light: 12 hour dark cycle in temperature and light-controlled room in the animal facility at the Pharmacology Department, University of Utrecht. Animals had *ad libitum* access to tap water and 150 g food was provided on alternate days. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

5.2.1.2. Diets

Diets used in this experiment were different from those used previously in order to determine the effect of ALA and LA on allergic sensitisation. The high-fat experimental diets - ALA- and LA-rich diets - and the low-fat soybean oil-based chow (AI*N-93*) were obtained from Research Diet Services B.V. (Wijk bij Duurstede, Netherlands). Experimental diets were produced by replacing 3 % of the wheat content of AI*N-93* with a fat blend high in LA or ALA. Macronutrient composition of the diets is shown in Table 5-2.

Table 5-2: Macronutrient composition of AI*N-93* (low fat), experimental (high fat) and "Western" diets. Taken from Research Diet Services [237].

Component	AI*N-93*	Experimental diets	"Western" diet
Carbohydrate (g / 100 g)	64	60	60
Protein (g / 100 g)	20	20	20
Fat (g / 100 g)	7	10	10
Fibre (g / 100g)	5	5	5
Mineral Mix (mg / 100 g)	35	35	35
Vitamin Mix (mg / 100 g)	10	10	10

The fatty acid content of diets is shown in Appendix Four and fatty acids of interest are shown in Figure 5-1.

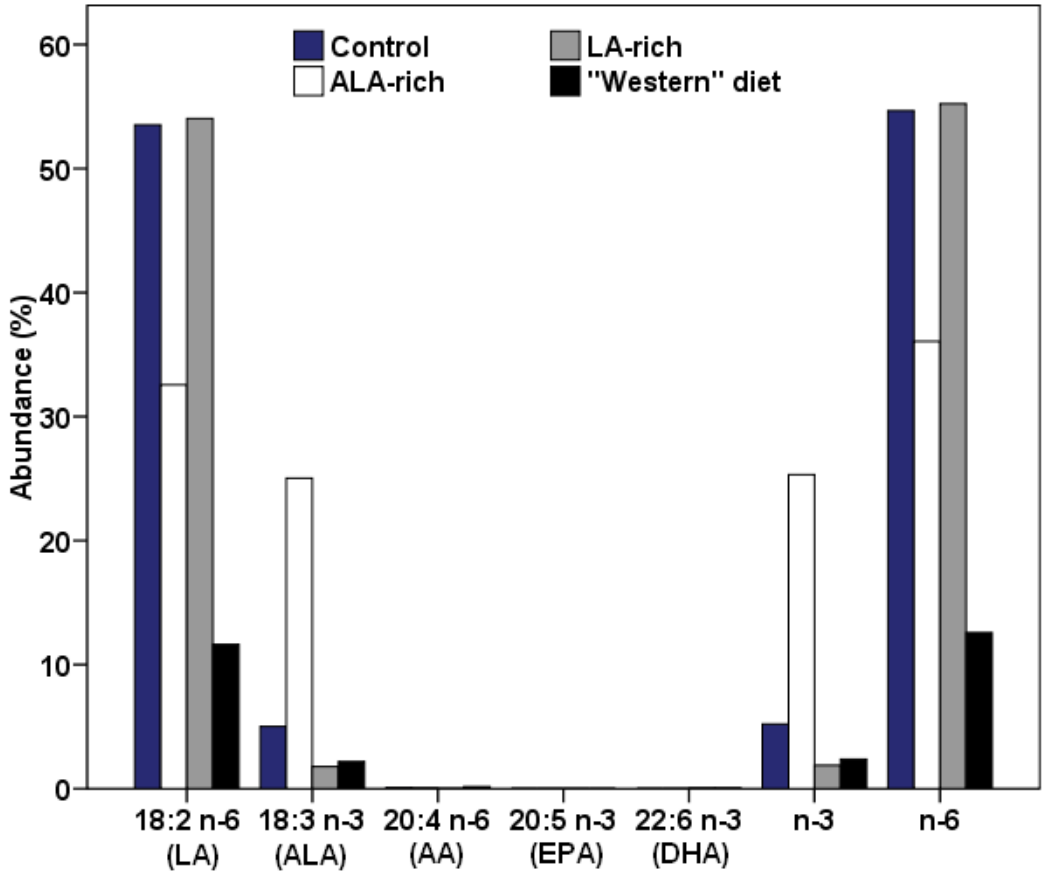

Figure 5-1: Profile of fatty acids of interest, total *n-3* and *n-6* PUFA contents.

The control diet had a high percentage of LA and *n-6* PUFAs, and low ALA, AA, EPA and total *n*-3 PUFAs. The ALA diet had a high percentage of ALA and *n*-3 PUFAs, but a low percentage of LA, AA, EPA, DHA and *n*-6 PUFAs. The LA diet was rich in LA, and total *n-6* PUFAs, but low in ALA and *n-3* PUFAs. The "Western" diet had a low LA, ALA, AA, EPA, DHA, *n-3* and *n-6* content, but was high in SFAs and MFAs.

5.2.1.3. Chemicals

Sources of chemicals were identical to those used previously (3.2.1.3).

5.2.2. Methods

An overview of the trial is provided in Figure 5-2. Sixteen 8-week old BALB/C female mice were randomised to one of seven dietary groups, and bred with age-matched controls. The dietary groups were a control diet (3% fat, containing soybean oil), ALA-rich diet in gestation only, ALA-rich diet in gestation and lactation, ALA-rich diet in lactation only, LA-rich diet in gestation only, LA-rich diet in gestation and lactation, LA-rich diet in lactation only. Animals that received the ALA- or LA-rich diet in either gestation or lactation alone were transferred to the control diet in the alternate period. At

21 days old, male pups were weaned onto a "Western" diet (rich in SFAs and MFAs, low in LA and ALA). Blood samples were taken from both dams and pups at this period by sampling from the cheek.

Pups were sensitised to ovalbumin at days 43 and 50. Acute skin tests were carried out on day 63. Mice (offspring) were challenged on days 71, 74, and 77 ± 1. Airway responsiveness was measured on day 78 ± 1. Euthanisation occurred by *intra peritoneal* injection of 1 ml 10 % urethane on day 78 ± 1.

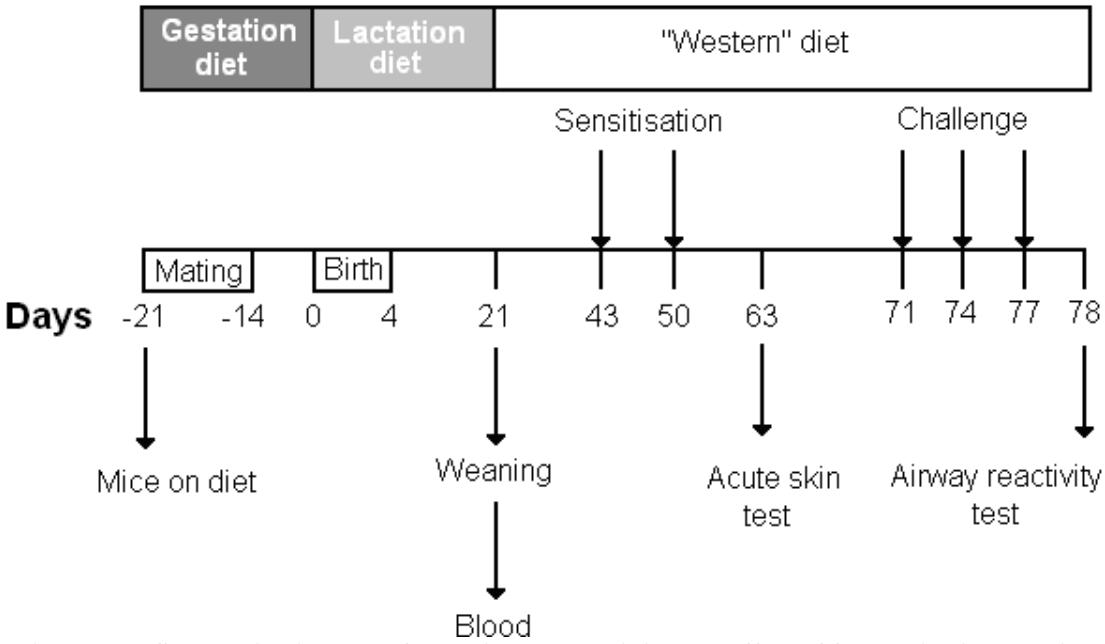

Figure 5-2: Schematic diagram of experiment examining the effect of fatty acids in gestation and / or lactation on a mouse model of allergic asthma.

5.2.2.1. Sensitisation of Mice to Ovalbumin

Mice were sensitised with ovalbumin at days 43 and 50, using the method described in 3.2.2.1.

5.2.2.2. Acute Skin Test to Ovalbumin Challenge

An acute skin test was carried out at day 63, using the method described in 3.2.2.2.

5.2.2.3. Determination of Airway Responsiveness to Ovalbumin

Mice were challenged with aerosolised ovalbumin at days 71 ± 1, 74 ± 1, 77 ± 1, and airway responsiveness to methacholine measured on day 78 ± 1, using the method described in 3.2.2.3.

5.2.2.4. Fatty Acid Analysis of Diet and Erythrocytes

At euthanisation, blood was collected, and erythrocytes separated, and together with samples of diet were analysed for fatty acids using the method previously described (2.2.2.3).

5.2.2.5. Analysis of Plasma Immunoglobulin Concentrations

Plasma, collected at euthanisation, was collected and analysed for IgG1, IgG2a and IgE using sandwich ELISAs, following the manufacturer's instructions.

5.2.2.6. Analysis of Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) was collected and analysed as described previously (3.2.2.6).

5.2.2.7. Determination of IL-5 Content of BALF

BALF was analysed for IL-5 content using the method described previously (3.2.2.7).

5.2.2.8. Cytospin Analysis of BALF

BALF cells were collected and analysed as described previously (3.2.2.8).

5.2.2.9. Analysis of Immune Tissue, Fat and Liver

Spleen, thymus, epididymal fat pads and liver were removed and weighed. Bone marrow was collected as described previously (2.2.2.5).

5.2.2.10. Histological Analysis of the Thymus

Sections of the thymus were analysed for cortex / medulla ratio as described previously (2.2.2.6).

5.2.2.11. Determination of Cytokine Production by Splenocytes

Splenocytes were prepared and cultured as described in 2.2.2.7. IFN-γ, IL-4 and IL-10 production in response to ovalbumin and αCD3 by spleen cells were analysed using the method described in 3.3.7.

5.2.2.12. Determination of Cell Subsets in Lymphoid Tissues using Flow Cytometry

Single cell suspensions of spleen, thymus and bone marrow were prepared and analysed for cell surface marker expression following the methods described in 2.2.2.8.

5.2.2.13. Statistical Analysis

Results were analysed using SPSS version 15.0. Parameters were analysed using Levene's test of homogeneity to ensure groups had equal variances, in which case they were compared using one-way analysis of variance (ANOVA), followed by *post-hoc* testing (Tukey). If Levene's test showed unequal variances to be present, groups were compared using the Kruskal-Wallis test. Where this was used, it is reported in the results section. If the Kruskal-Wallis test indicated a significant effect, Mann-Whitney U independent group comparisons tests were then carried out to compare each combination of groups. Analysis of fatty acid content of erythrocytes at weaning and euthanisation was compared using two-way ANOVA. If variances were present, fatty acid content of dietary groups was compared using one-way ANOVA, followed by *post-hoc* testing (Tukey).

5.3. Results

5.3.1. Gestational Data

Data on diet in gestation on numbers of offspring, males, females or ratio of males to females are shown in Table 5-3. There was no effect of diet on any of the parameters measured.

Table 5-3: Effect of diet in gestation on number of offspring, males, females and ratio of males to females. Ratio of males to females was analysed using non-parametric testing. There was no effect of diet on any of the parameters measured.

	Control (n = 7)	ALA-rich (n = 6)	LA-rich (n = 4)
Offspring (n)	8.1 ± 0.5	6.5 ± 0.8	7.0 ± 1.1
Males (n)	2.7 ± 0.4	2.7 ± 0.8	2.8 ± 0.5
Females (n)	5.4 ± 0.5	3.8 ± 1.3	4.3 ± 1.4
Ratio of males to females	0.6 ± 0.1	1.9 ± 1.0	1.2 ± 0.6

5.3.2. Body Weights of Offspring of Mice fed the Different Diets

Changes in body weight in offspring mice from different dietary groups are shown in Figure 5-3.

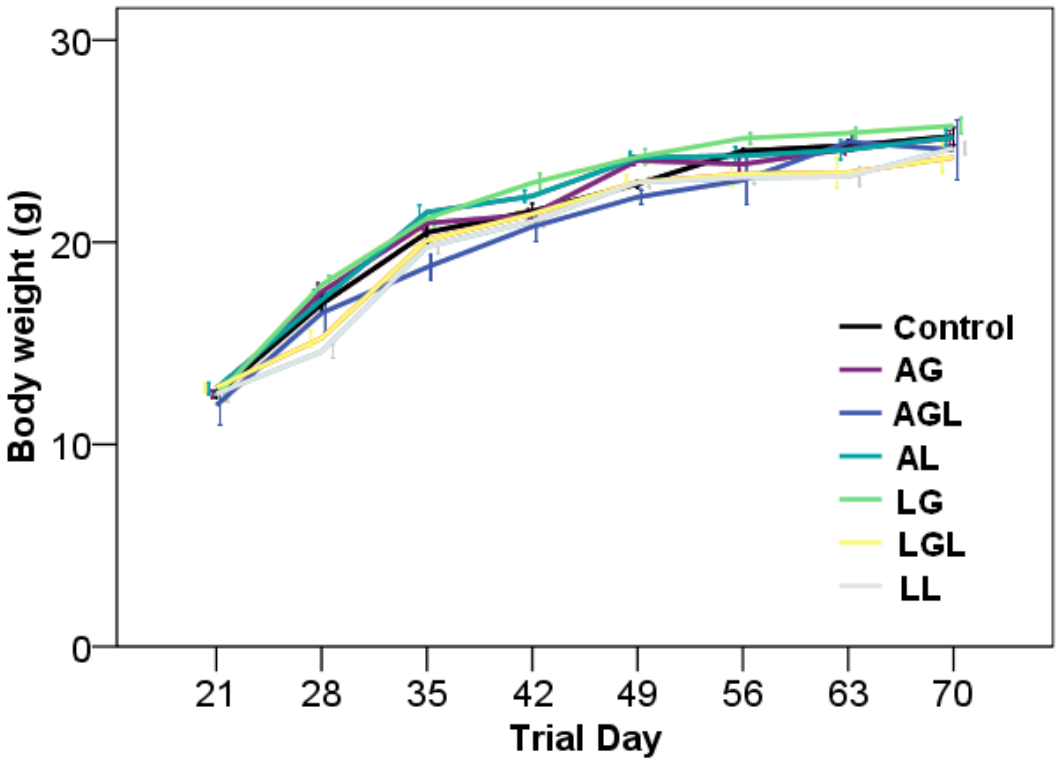

Figure 5-3: Body weight of offspring mice during the trial. Mice were born from day 0, weaning took place at day 21.

At day 28, the AG and LG groups were heavier than the LL and LGL groups. At day 35, the AL, LG and AG groups were heavier than the AGL group. At day 42 the LG group was heavier than the AGL and LL groups. At day 49, the AG, AL and LG groups were heavier than the AGL group. At day 56 the LG group was heavier than the AGL group. At day 63 the LG group was heavier than the LL group. There were no differences in body weights between dietary groups at days 21, 70 or 78 ± 1 (day of euthanisation; data not shown).

5.3.3. Tissue weights of Offspring of Mice fed the Different Diets

Tissue weights of mice fed the different experimental diets are shown in Table 5-4. Brains were lighter in the control group than all groups receiving high fat diets in gestation and / or lactation, when data was expressed both as an absolute value and in proportion to body weight. There were no differences in weight of immune tissues between dietary groups.

Table 5-4: Effect of diet on tissue weights of mice. Values with different letters are statistically different from one another (p < 0.0 body weight) was analysed using non-parametric testing.

	Control (n = 5)	AG (n = 9)	AGL (n = 3)	AL (n = 7)	LG (n = 5)	L (n
Spleen (g)	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.16 ± 0.02	0.15 ± 0.01	0.16
Spleen (% of body weight)	0.53 ± 0.05	0.58 ± 0.03	0.63 ± 0.04	0.61 ± 0.03	0.57 ± 0.02	0.63
Thymus (g)	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04
Thymus (% of body weight)	0.15 ± 0.02	0.14 ± 0.01	0.10 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.18
Liver (g)	1.52 ± 0.05	1.47 ± 0.03	1.49 ± 0.03	1.43 ± 0.06	1.47 ± 0.03	1.30
Liver (% of body weight)	5.72 ± 0.10	5.62 ± 0.08	5.66 ± 0.29	5.66 ± 0.22	5.47 ± 0.11	5.17
Epididymal fat (g)	0.49 ± 0.07	0.50 ± 0.03	0.48 ± 0.03	0.46 ± 0.06	0.44 ± 0.01	0.53
Epididymal fat (% of body weight)	2.07 ± 0.22	1.90 ± 0.11	1.85 ± 0.20	1.94 ± 0.30	1.63 ± 0.04	2.04
Brain (g)	3.96 ± 0.11[a]	4.48 ± 0.07[b]	4.68 ± 0.14[b]	4.59 ± 0.08[b]	4.78 ± 0.12[b]	4.57
Brain (% of body weight)	14.98 ± 0.61[a]	17.15 ± 0.31[b]	17.71 ± 0.78[b]	18.23 ± 0.53[b]	17.83 ± 0.51[b]	18.19

5.3.4. Fatty Acid Content of Erythrocytes of Offspring of Mice fed the Different Diets

Erythrocytes from offspring taken at weaning and euthanisation were analysed for fatty acid content. Data are shown in detail in Appendix Four and data for fatty acids of interest are shown in Figure 5-4.

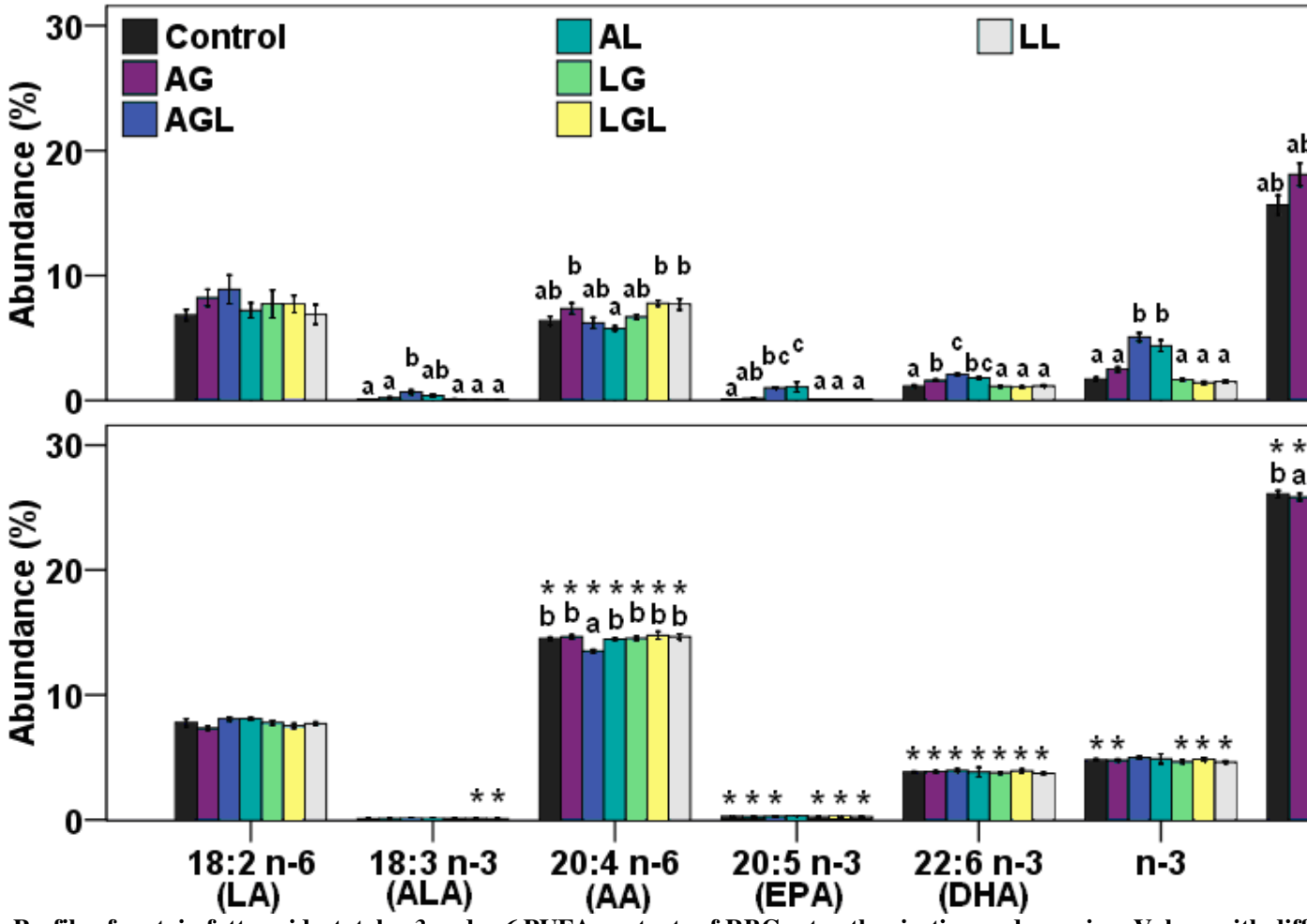

Figure 5-4: Profile of certain fatty acids, total *n-3* and *n-6* PUFA contents of RBCs at euthanisation and weaning. Values with diff in fatty acid abundance between dietary groups. Asterisks denote differences in fatty acids between time p

At weaning, ALA content of erythrocytes was higher in the AGL group than the control, AG, LG, LGL and LL groups. The AA content was higher in the AG, LGL and LL groups than in the AL group at weaning. EPA content was higher in the AL group than all groups except AGL at weaning; EPA content in the AGL group was higher than in the control and groups receiving the LA-rich diet. At weaning, DHA content was higher in the AGL group than all other dietary groups with the exception of the AL group; DHA content was higher in groups receiving the ALA-rich diet than those on the control or LA-rich diet. At weaning, *n*-3 PUFA content was highest in the AGL and AL groups. *n*-6 PUFA content at weaning was higher in the LGL group than the AL group.

At euthanisation, when mice had been receiving the "Western diet" for 57 ± 1 days, AA content was lower in the AGL group than all other groups, and *n*-6 content was lower in the AG and AGL groups than all other groups. Differences in ALA content between blood samples taken at weaning, and those taken at euthanisation were observed in the LGL and LL groups. AA content was higher in all groups at euthanisation compared to weaning. EPA content was higher in all groups at euthanisation except for the AL group, where no difference was observed. DHA and *n-6* content were higher at euthanisation than weaning for all groups. *n*-3 content was higher in all groups except for the AGL and AL groups at euthanisation compared to weaning.

5.3.5. Airway Responsiveness in the Offspring of Mice fed the Different Diets

Airway responsiveness data is shown in Figure 5-5. There was no effect of maternal diet on airway responsiveness to methacholine in ovalbumin-sensitised mice. However, SEMs were large, and there may have been differences that were obscured due to this, and that may have been apparent if sample sizes were higher.

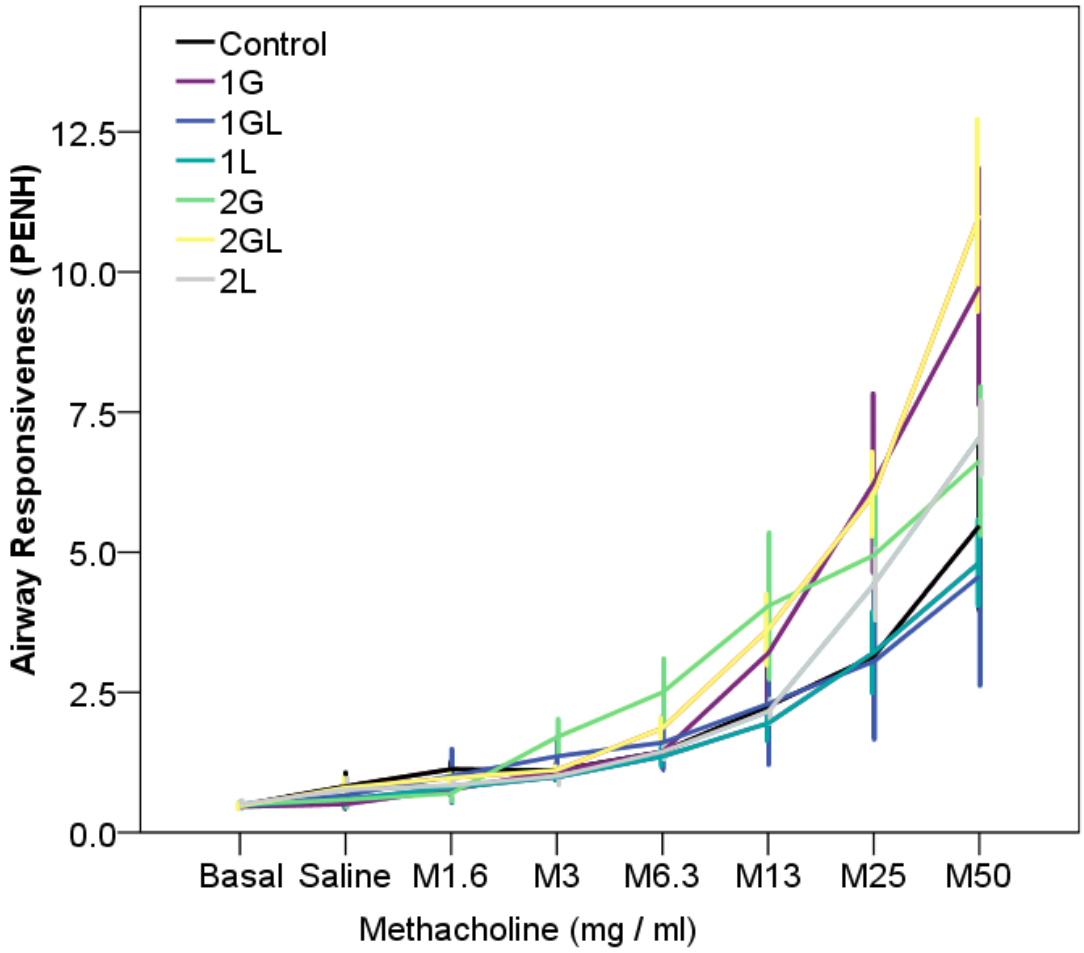

Figure 5-5: Effect of diet on airway responsiveness to increasing doses of methacholine at day 78 of the trial.

5.3.6. Acute Skin Test in Offspring of Mice fed the Different Diets

The effect of the experimental diets on ear swelling in response to ovalbumin is shown in Figure 5-6.

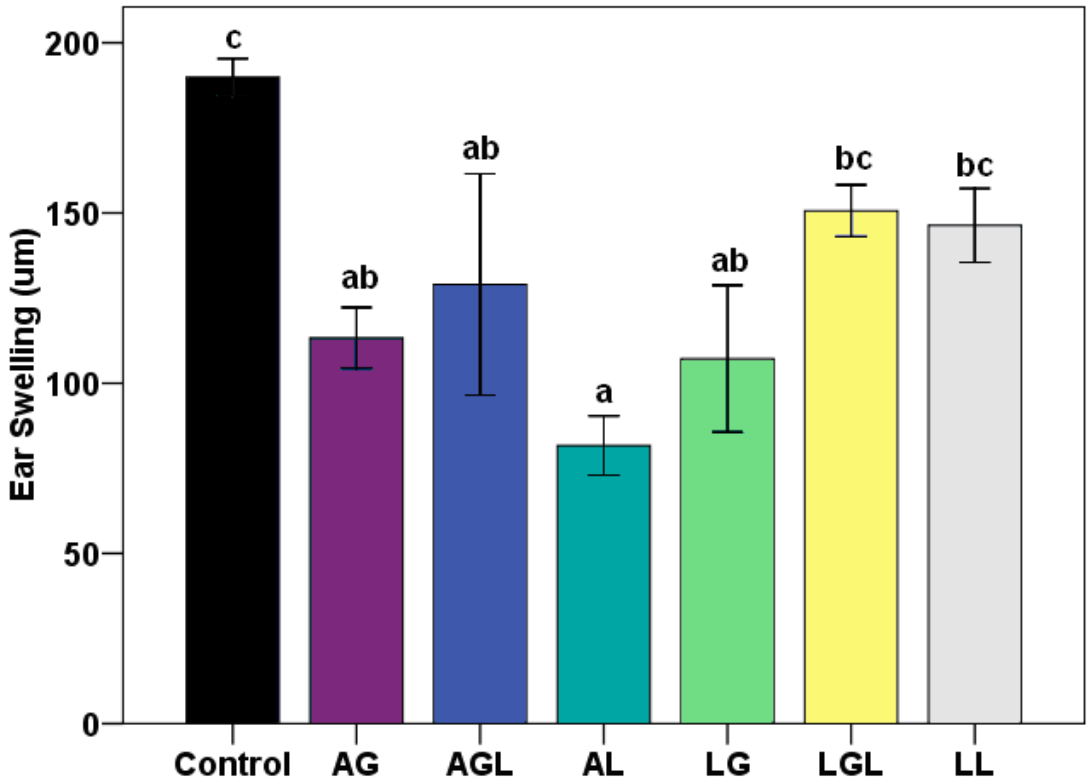

Figure 5-6: Effect of maternal diet on ear swelling in response to ovalbumin in ovalbumin-sensitive offspring. Bars with different letters are statistically different from one another ($p < 0.05$).

Ear swelling in response to ovalbumin was lower in animals whose dams had received the ALA-rich in gestation and / or lactation, and those receiving the LA-rich diet in gestation compared to those receiving the low fat control diet for the whole period. Animals in the AL group had a lower ear swelling response than the LGL and LL groups.

5.3.7. Plasma Immunoglobulin Concentrations in Offspring of Mice Fed the Different Diets

Antibody concentrations of plasma is shown in Figure 5-7 (IgE), Figure 5-8 (IgG1) and Figure 5-9 (IgG2a). There was no effect of diet in gestation or lactation on any of the parameters measured.

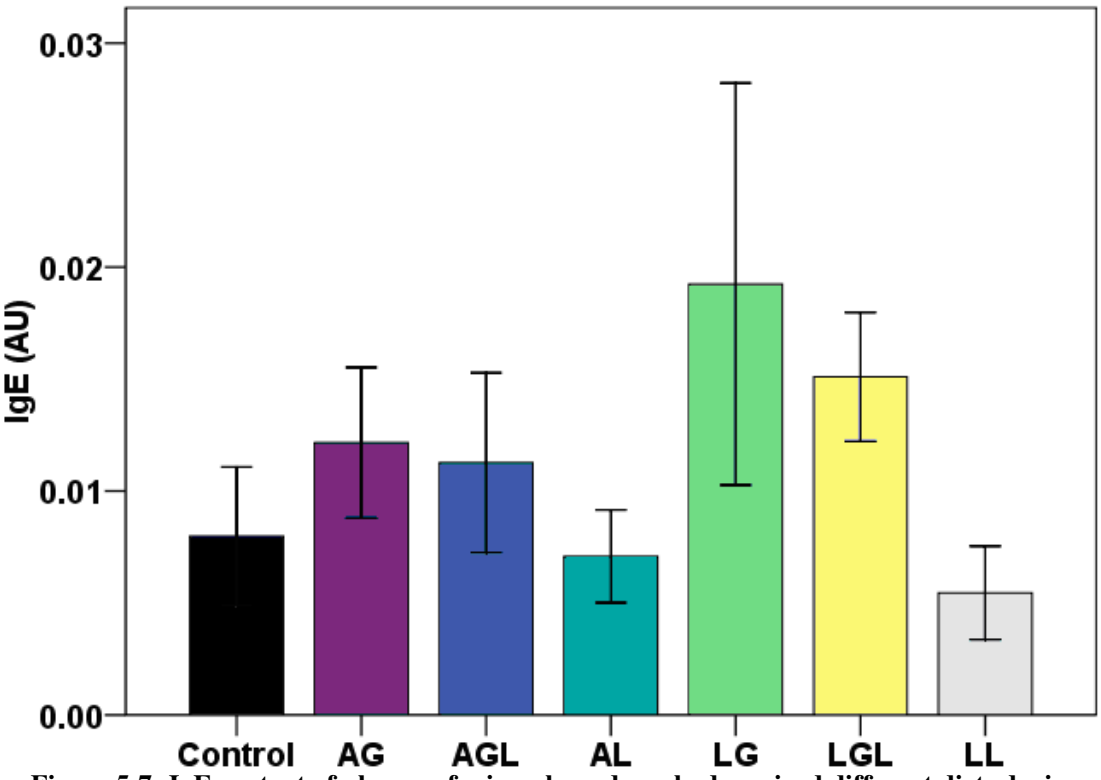

Figure 5-7: IgE content of plasma of mice whose dams had received different diets during gestation and / or lactation. There was no significant difference between groups. Data was analysed using non-parametric testing.

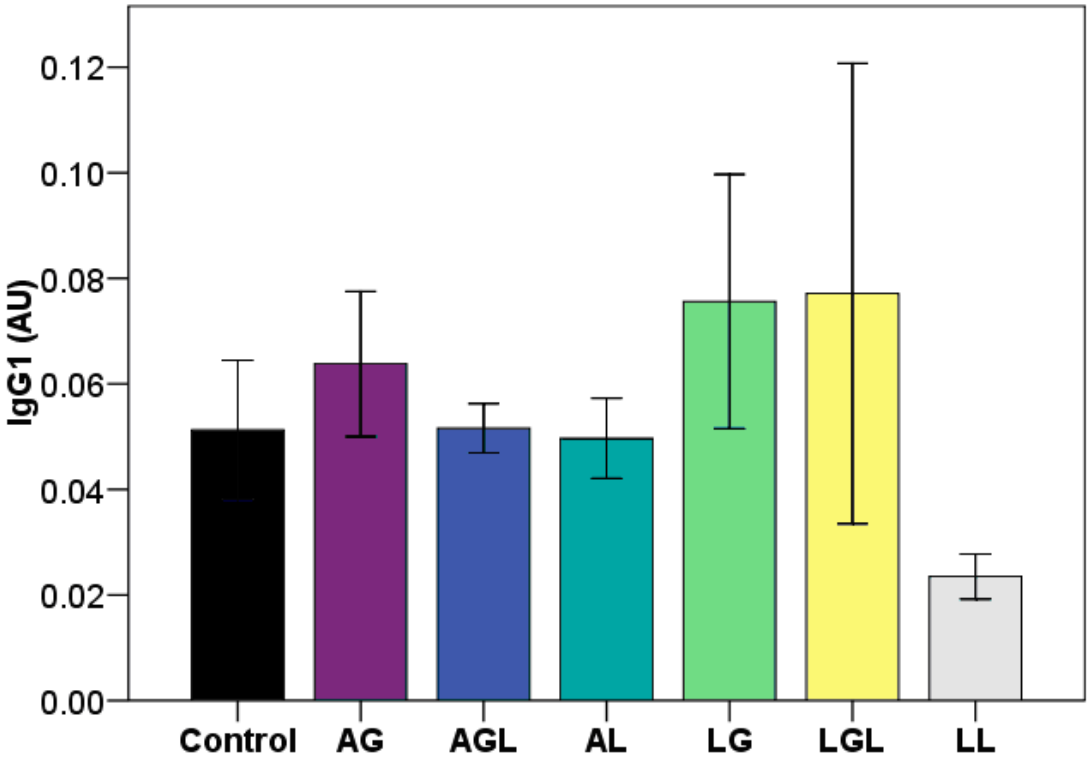

Figure 5-8: IgG1 content of plasma of mice whose dams had received different diets during gestation and / or lactation. There was no significant difference between groups. Data was analysed using non-parametric testing.

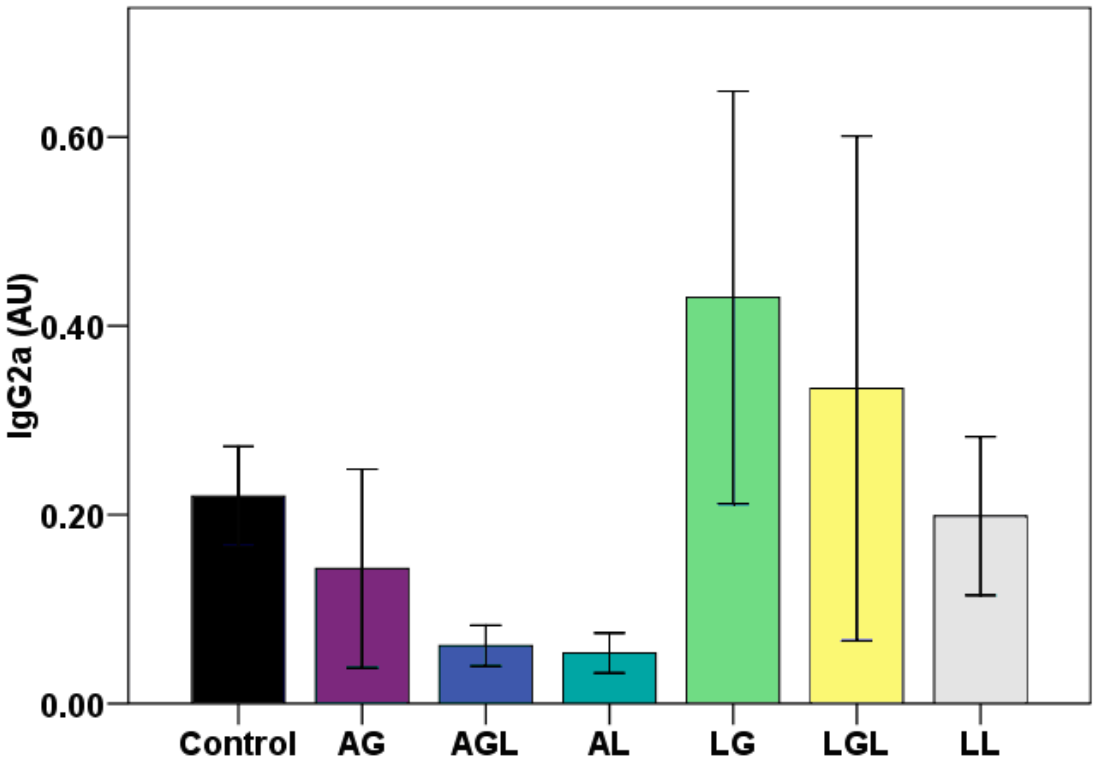

Figure 5-9: IgG2a content of plasma of mice whose dams had received different diets during gestation and / or lactation. There was no significant difference between groups. Data was analysed using non-parametric testing.

5.3.8. Cytokine Production by Cultured Splenocytes from Offspring of Mice Fed the Different Diets

Anti-CD3- and OVA-stimulated production of IL-4, IL-10, IL-5 and IFN-γ production of cultured spleen cells is shown in Figure 5-10 to Figure 5-17. There was no effect of diet on any of the parameters measured, but there was large variation observed.

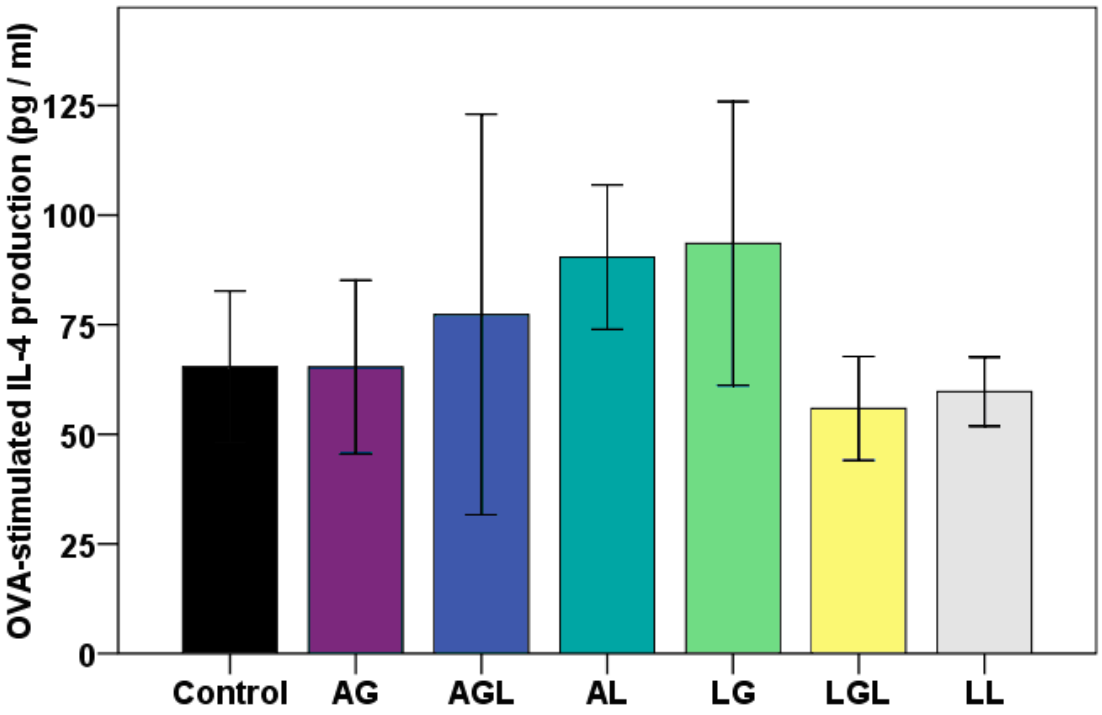

Figure 5-10: Effect of diet on OVA-stimulated IL-4 production in spleen cells. There was no significant difference between dietary groups.

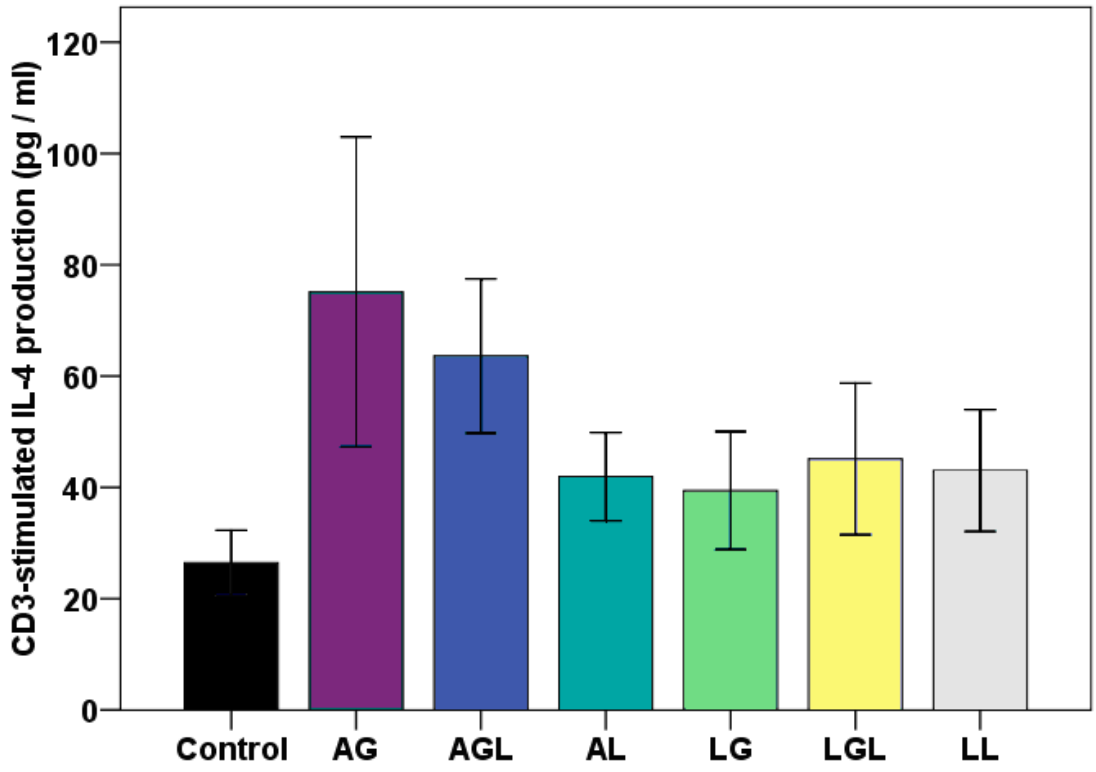

Figure 5-11: Effect of diet on CD3-stimulated IL-4 production in spleen cells. There was no significant difference between dietary groups. Data were analysed using non-parametric testing.

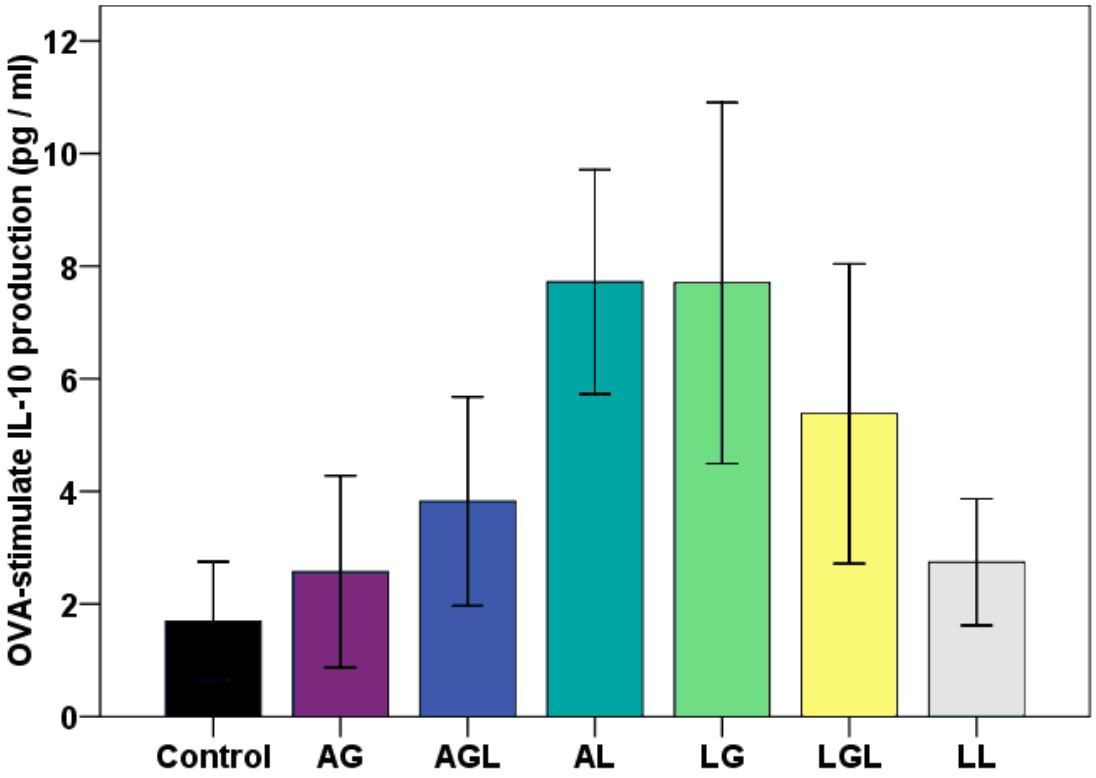

Figure 5-12: Effect of diet on OVA-stimulated IL-10 production in spleen cells. There was no significant difference between dietary groups.

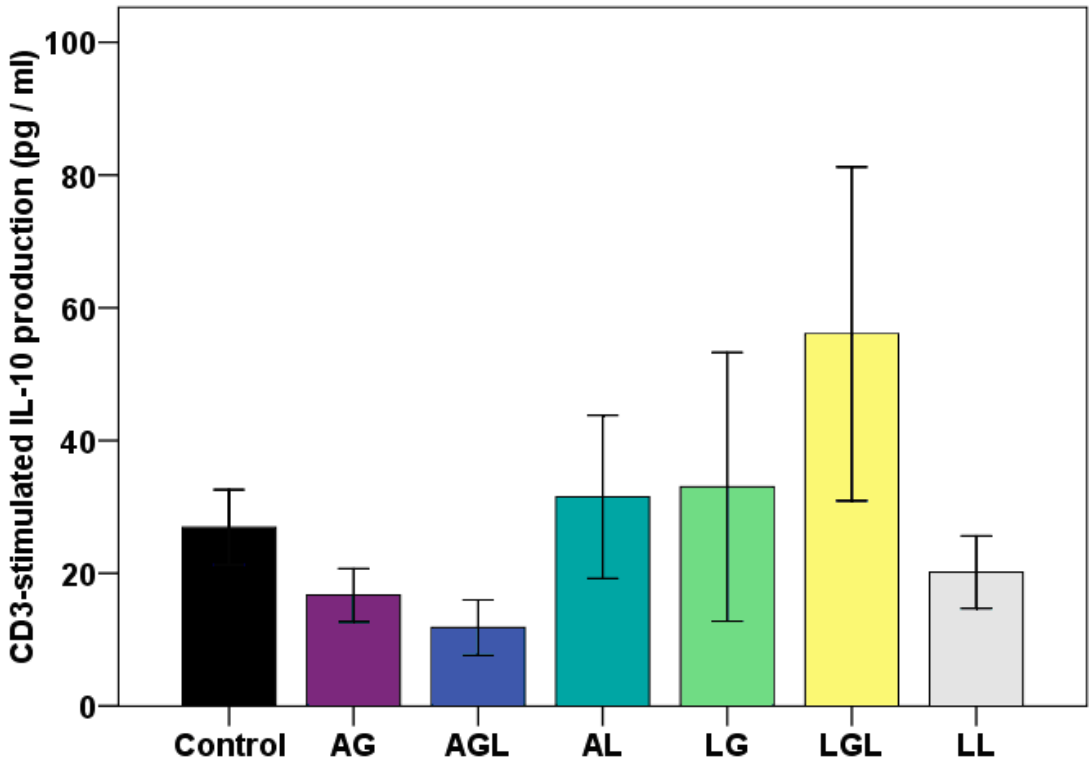

Figure 5-13: Effect of diet on CD3-stimulated IL-10 production in spleen cells. There was no significant difference between dietary groups. Data was analysed using non-parametric testing.

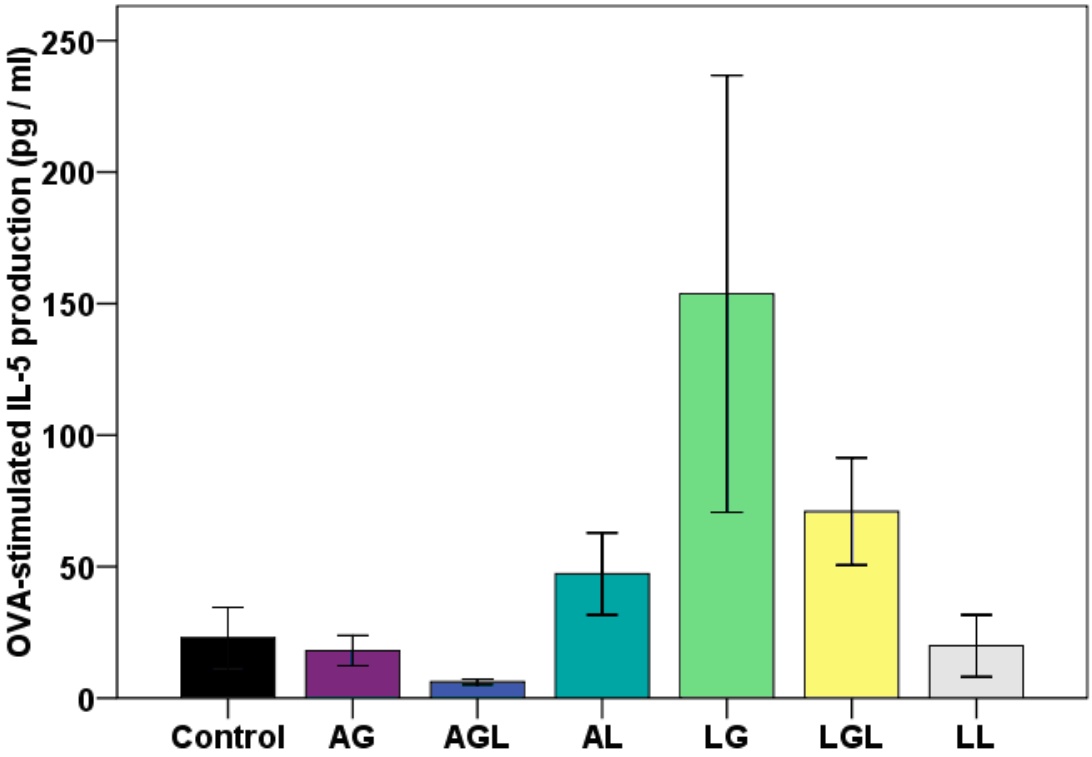

Figure 5-14: Effect of diet on OVA-stimulated IL-5 production in spleen cells. There was no significant difference between dietary groups. Data was analysed using non-parametric testing.

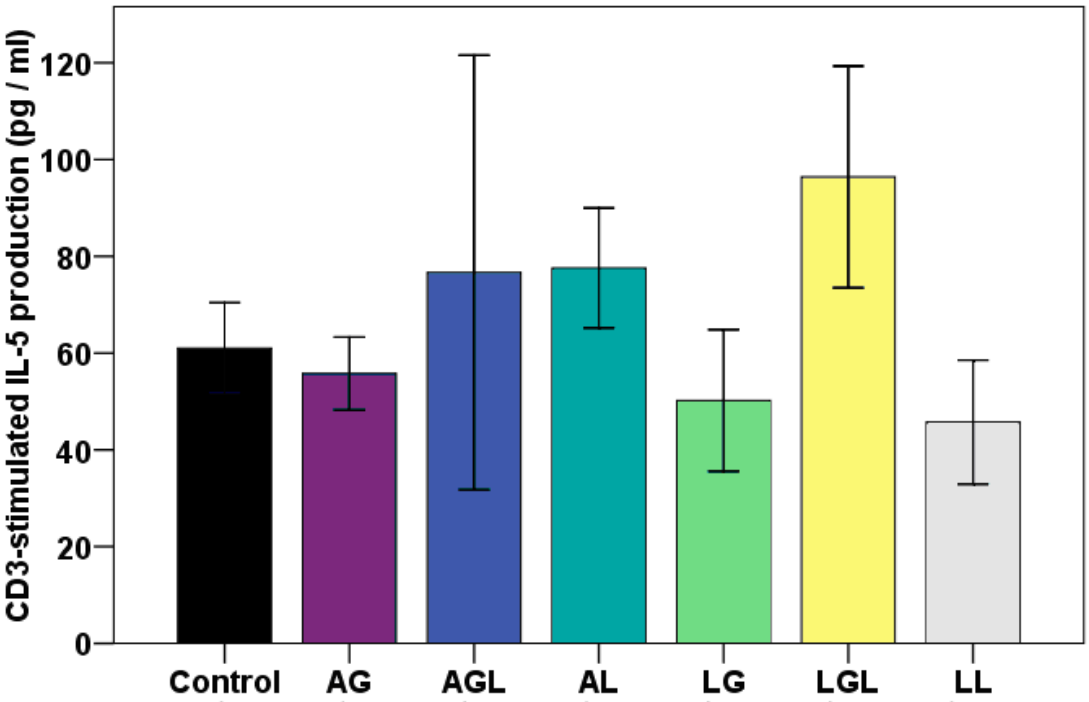

Figure 5-15: Effect of diet on CD3-stimulated IL-5 production in spleen cells. There was no significant difference between dietary groups.

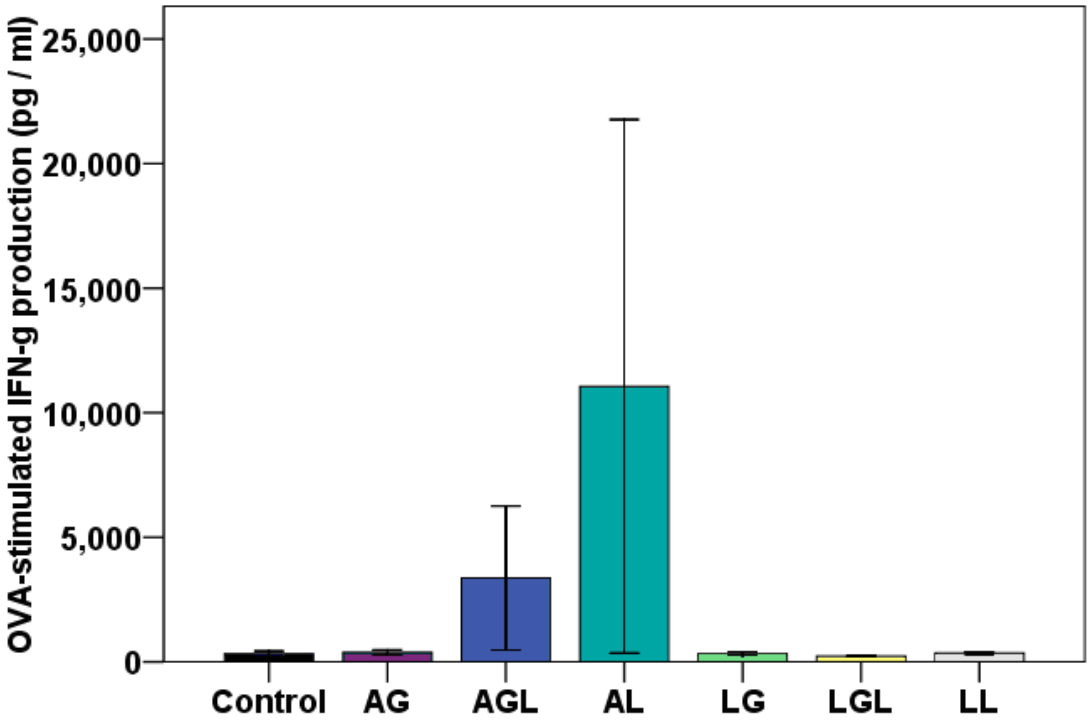

Figure 5-16: Effect of diet on OVA-stimulated IFN-γ production in spleen cells. There was no significant difference between dietary groups. Data was analysed using non-parametric testing.

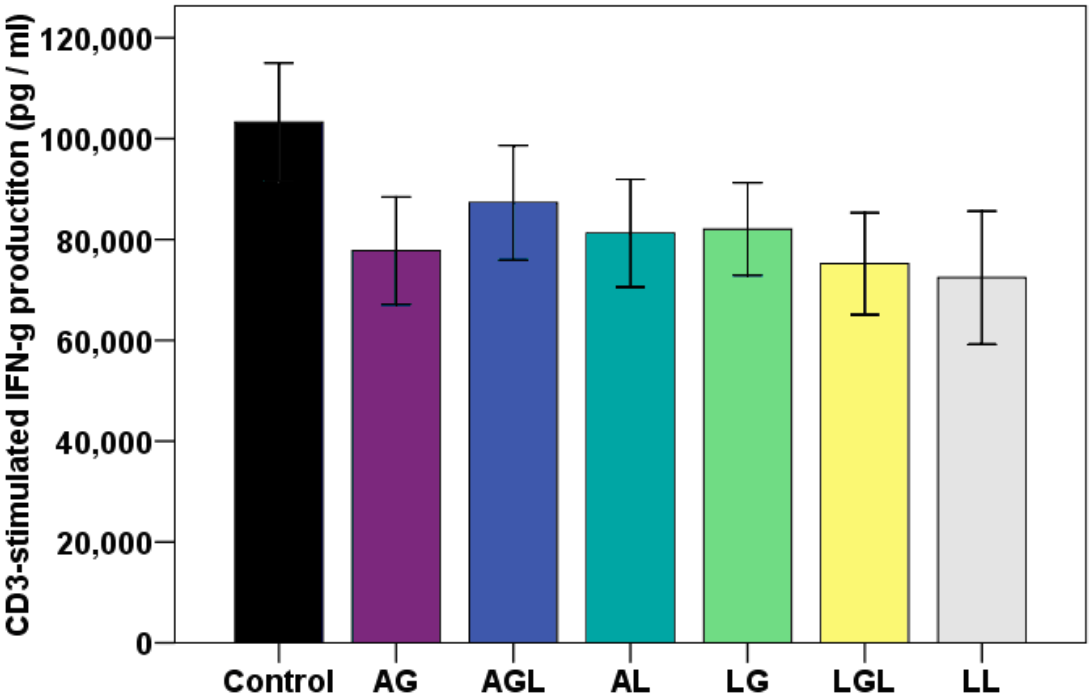

Figure 5-17: Effect of diet on CD3-stimulated IFN-γ production in spleen cells. There was no significant difference between dietary groups.

5.3.9. IL-5 Content of BALF from Offspring of Mice Fed Different Diets

IL-5 content of BALF is shown in Figure 5-18. There was no significant difference between dietary groups.

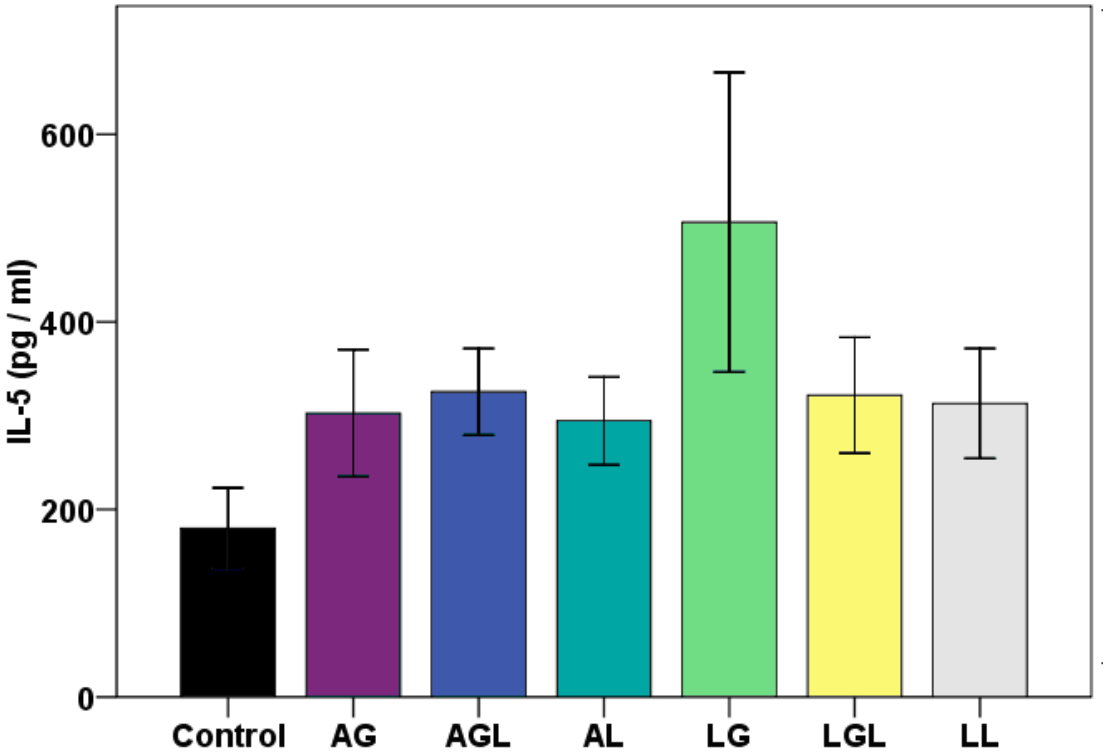

Figure 5-18: IL-5 content of bronchoalveolar lavage fluid. There was no significant difference between any dietary groups.

5.3.10. Cytospin Analysis of BALF from Offspring of Mice Fed the Different Diets

Results from the cytospin analysis of the bronchoalveolar lavage fluid (BALF) are shown in Figure 5-19. There was no effect of diet in gestation or lactation on any of the parameters measured.

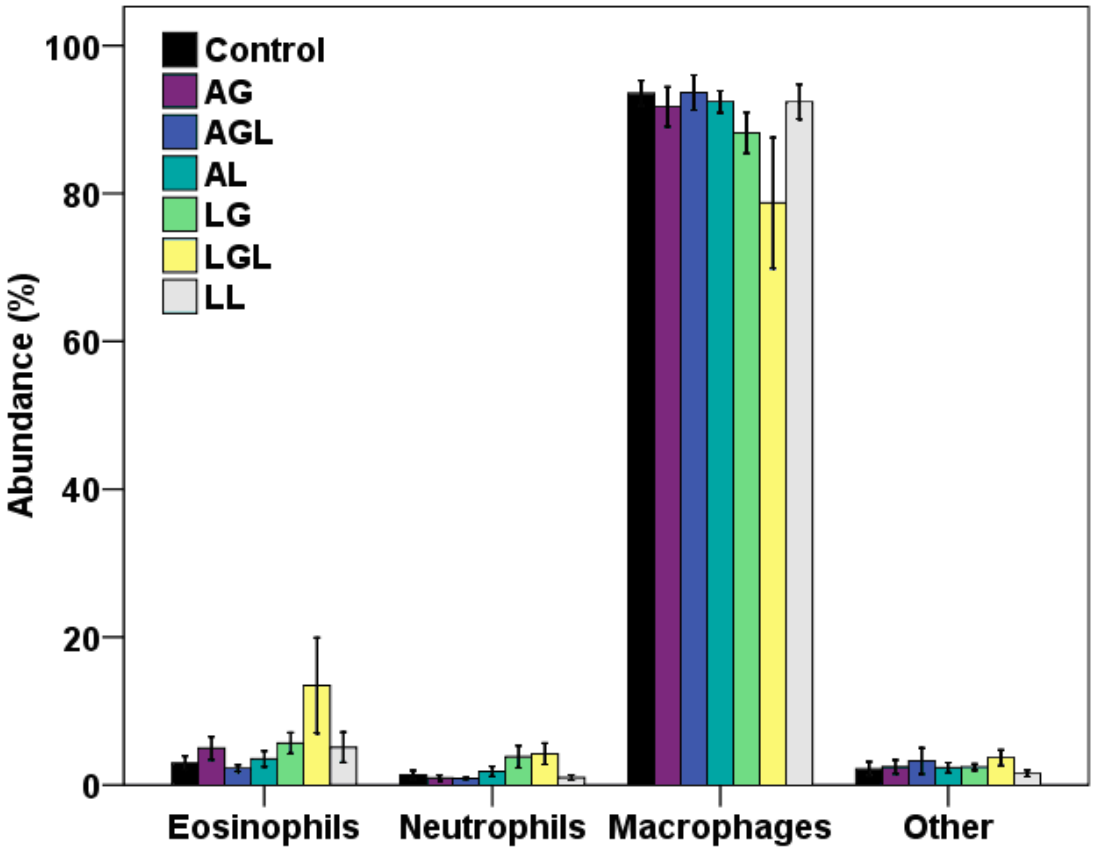

Figure 5-19: Cytospin analysis of bronchoalveolar lavage fluid. There was no effect of diet on any of the parameters measured.

5.3.11. Histological Analysis of Thymuses from Offspring of Mice Fed the Different Diets

The cortex / medulla ratio of mice thymuses is shown in Figure 5-20. There were no significant differences between dietary groups.

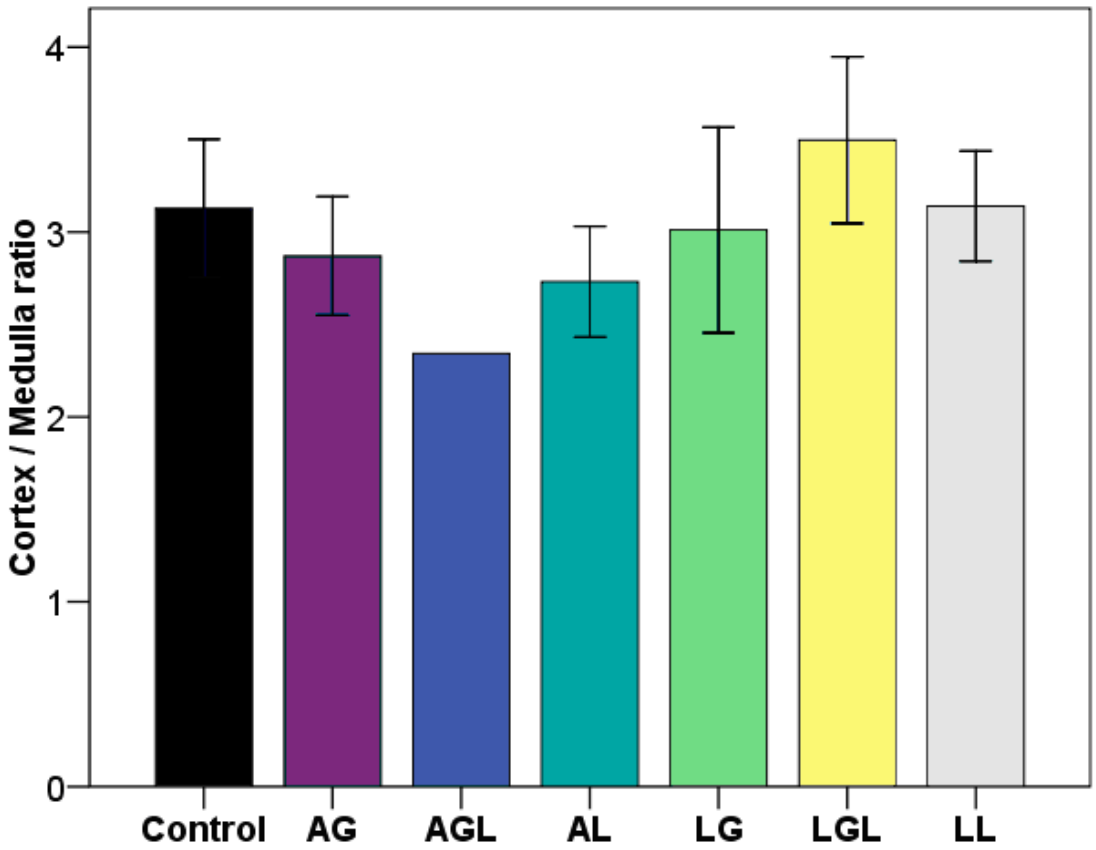

Figure 5-20: Cortex / Medulla ratio of thymuses. There were no significant differences as a result of diet. Due to errors in data collection, only one sample was usable for analysis in the AGL group.

5.3.12. Cell Subsets in the Thymus, Spleen and Bone Marrow from Offspring of Mice Fed the Different Diets

Effect of diet on expression of cell surface markers of the thymus is shown in Table 5-5. CD3+ expression in thymus cells was lowest in the control and AG groups, and higher in the LG, LGL and LL groups than in the AL group. CD3+ cells that also expressed CD4+ cells were lowest in the LG and LGL groups, and highest in the AGL and LL groups. CD3+ cells that also expressed CD8+ in the thymus were lower in the control, AG, AGL and AL groups than in the LG and LGL groups. There was no effect of diet on CD3+CD4+ cells that were also CD69+. Expression of CD62L+ on CD3+CD4+ thymus cells was higher in the control and AG groups than all other groups. CD4+CD25+ expression in thymus cells was higher in the AG and LL groups than the AGL and AL groups. Expression of FoxP3+ in the CD4+CD25+ thymus cells was lowest in the control group, and highest in the LG, LGL and LL groups.

Table 5-5: Effect of diet on the expression of cell surface markers of the thymus. All parameters were analysed using non-para different letters denote differences in cell abundance between dietary groups.

	Cells Identified	Control (n = 5)	AG (n = 9)	AGL (n = 3)	AL (n = 7)	LG (n = 5)
CD3+	T-cells	81.29 ± 1.58^{a}	77.59 ± 4.63^{a}	94.71 ± 1.30^{bcd}	94.12 ± 0.46^{b}	97.10 ± 0.80^{cd}
CD3+ that are also CD4+	T_H cells	91.87 ± 0.36^{b}	93.71 ± 0.68^{b}	97.52 ± 0.05^{c}	97.51 ± 0.05^{c}	84.05 ± 0.98^{a}
CD3+ that are also CD8+	CTLs	83.25 ± 1.34^{ab}	73.96 ± 7.07^{a}	82.66 ± 0.56^{ab}	83.51 ± 1.47^{ab}	87.82 ± 0.95^{c}
CD3+CD4+ that are also CD62L+	L-selectin expression	95.21 ± 0.67^{c}	85.13 ± 4.69^{c}	64.66 ± 2.02^{ab}	67.51 ± 1.07^{b}	64.47 ± 1.00^{a}
CD4+CD25+	T-regs	4.01 ± 0.83^{abcd}	4.30 ± 0.52^{d}	1.77 ± 0.05^{a}	2.24 ± 0.16^{b}	2.14 ± 0.21^{ab}
CD4+CD25+ that are also FoxP3+	T-regs	8.74 ± 1.08^{a}	10.92 ± 2.48^{ab}	19.14 ± 1.42^{bc}	13.97 ± 3.04^{b}	20.14 ± 1.39^{c}

Effect of diet on expression of cell surface markers of the spleen is shown in Table 5-6. F4-80+ (macrophage) expression in the spleen was highest in the AL group, and lowest in the control, AG, LG and LL groups. There was no difference in CD3+ expression between dietary groups. CD3+ cells that also expressed CD4+ abundance was highest in the AGL, AL and LL groups; these groups also had the lowest expression of CD8+. CD3+CD4+ cells that also expressed CD62L+ abundance was lower in the LG and LGL groups than the LL group. CD3+CD4+ cells that were CD69+ were lowest in the LL group, and higher in the control group than the AGL and LGL groups. CD19+ expression was lower in the AGL, AL and LL groups than the control and AG groups. CD4+CD25+ expression was lower in the AL group than the AGL, LGL and LL groups, and lower in the LGL group than the LL group. FoxP3+ expression in CD4+CD25+ cells was lower in the control and AG groups than the AGL, AL, and LL groups. CD94+ expression was lower in the control group than in the LL group.

Effect of diet on expression of cell surface markers of bone marrow is shown in Table 5-7; this was the only cell type measured in bone marrow. There was no effect of diet on CD19+ expression in bone marrow.

Table 5-6: Effect of diet on the expression of cell surface markers of the spleen. Data is not available for CD19+ in the 2G and 2G formulation. CD3+CD8+, CD3+CD4+CD69+ CD4+CD25+, and CD4+CD25+FoxP3+ were analysed using non-parametric testing denote differences in cell abundance between dietary groups.

	Cells Identified	Control (n = 5)	AG (n = 9)	AGL (n = 3)	AL (n = 7)	LG (n = 5)	
F4-80	Macrophages	6.35 ± 0.63^{a}	7.23 ± 1.09^{ab}	14.60 ± 1.77^{c}	21.61 ± 1.13^{d}	8.93 ± 0.89^{ab}	
CD3+	T-cells	29.17 ± 1.76	28.51 ± 1.02	28.95 ± 1.69	28.21 ± 2.06	31.47 ± 0.61	
CD3+ that are also CD4+	T_H cells	54.15 ± 1.50^{a}	58.65 ± 2.35^{ab}	71.64 ± 2.08^{c}	64.49 ± 2.51^{bc}	51.07 ± 0.88^{a}	
CD3+ that are also CD8+	CTLs	14.93 ± 0.66^{d}	10.63 ± 1.88^{cd}	1.50 ± 0.07^{b}	1.47 ± 0.12^{ab}	2.65 ± 0.91^{bc}	
CD3+CD4+ that are also CD62L+	L-selectin expression	47.79 ± 2.90^{ab}	48.98 ± 2.73^{ab}	49.15 ± 2.96^{ab}	46.97 ± 1.13^{ab}	43.37 ± 2.22^{a}	
CD3+CD4+ that are also CD69+	Activated leukocytes	64.41 ± 2.20^{c}	73.36 ± 7.51^{bc}	42.14 ± 4.65^{b}	71.98 ± 8.36^{bc}	49.26 ± 5.20^{bc}	
CD19+	B-cells	16.16 ± 1.18^{b}	13.67 ± 1.23^{b}	8.25 ± 0.52^{a}	7.51 ± 0.67^{a}		
CD4+CD25+	T-regs	5.73 ± 0.79^{abc}	4.43 ± 0.75^{ab}	4.83 ± 0.16^{b}	4.16 ± 0.11^{a}	4.85 ± 0.39^{ab}	
CD4+CD25+ that are also FoxP3+	T-regs	4.52 ± 2.19^{a}	8.12 ± 3.71^{ab}	31.52 ± 4.52^{cd}	31.42 ± 1.37^{d}	24.37 ± 1.71^{bc}	
CD94+	NK cells	36.30 ± 2.58^{a}	44.55 ± 0.99^{ab}	41.84 ± 2.48^{ab}	41.86 ± 2.67^{ab}	44.70 ± 0.68^{ab}	

Table 5-7: Effect of diet on the expression of cell surface markers of bone marrow. There was no effect of diet on CD19+ expressio

	Cells Identified	Control (n = 5)	AG (n = 9)	AGL (n = 3)	AL (n = 7)	LG (n = 5)	LGL (n = 5)	L (n =
CD19+	B-cells	6.76 ± 0.84	13.80 ± 3.34	5.84 ± 0.82	9.48 ± 2.01	6.94 ± 4.00	8.59 ± 4.12	$12.44 \pm$

5.4. Discussion

5.4.1. Summary of findings

This study aimed to determine the effect of diets differing levels of ALA or LA given during gestation and / or lactation, on the immune response in a mouse model of allergy to the egg protein ovalbumin. The study set out to test hypotheses of reduced severity with ALA-rich diet in pregnancy / pregnancy and lactation and increased severity with LA-rich diet in pregnancy / pregnancy and lactation. It was hypothesised that increased ALA or LA intake in lactation would have less of an effect than increased ALA or LA intake in pregnancy.

Using ear swelling in response to ovalbumin, to which the mice had been sensitised, as the outcome, animals whose dams had received the ALA-rich in gestation and lactation, and those receiving the LA-rich diet in gestation had lower ear swelling compared to those receiving the low fat control diet for the whole period. Animals receiving the ALA-rich diet in lactation had lower ear swelling response than those receiving the LA-rich diet in gestation and lactation, or lactation alone. Despite this, there were no effects on lung function or immune markers measured in BALF, plasma, thymocytes, bone marrow or splenocytes.

Fatty acid analysis of diets showed that they contained the fatty acids expected; the control group had high a balance of *n-3* and *n-6* PUFAs, the LA-rich diet had high LA and *n-6* PUFAs, the ALA-rich diet had high ALA and *n-3* PUFAs, and the "Western" diet was rich in SFAs and MFAs. This was to the most part reflected in fatty acid analysis of erythrocytes taken at weaning, particularly in groups receiving experimental diets during lactation. By euthanisation, most of these differences in fatty acid composition of erythrocytes induced by diet had been lost.

5.4.2. Fatty Acid Content of Erythrocytes and Diets

Fatty acid content of diet was to the most part reflected in fatty acid analysis of erythrocytes taken at weaning, particularly in groups receiving experimental diets during lactation. Animals receiving the ALA-rich diets had higher levels of the long chain *n-3* PUFAs EPA and DHA, presumably because of reduced competition for the elongase and desaturase enzymes from *n-6* PUFAs. By euthanisation, most of these differences in fatty acid composition of erythrocytes induced by diet had been lost. The exceptions to this were the lower AA content of erythrocytes in the AGL group and the lower *n-6* PUFA content of the AG and AGL groups. The AA content of the AGL group may have been lower as

the lower-AA (ALA-rich) diet had been consumed for a longer period (six weeks) than the AG or AL groups (three weeks). Supplementing lactating women with flaxseed oil (10.7 g ALA per day) for six weeks reduced AA content of erythrocytes at end of supplementation, and four weeks after supplementation ceased compared to baseline values [238]. The reduction in AA content seen in women supplementing in lactation was not observed in the current study, which may have been due to the shorter period of supplementation, or the lower dose received by mice compared to that of the women. The *n-6* PUFA content being lower in the AG and AGL groups is lower due to the *n-3* rich diet received. It is possible that the AL group, in contrast to the other ALA-rich dietary groups, had not been receiving the diet long enough or perhaps too recently to have had erythrocytes affected by the diet. Fatty acid content of erythrocytes is a longer term reflection of fatty acid intake than fatty acid content of plasma, for example, because of a longer half life, and lower sensitivity to recent intake [239].

5.4.3. Gestational Data, Tissue and Body Weights

There was no difference in number of offspring, males or females, or the ratio of males to females between dietary groups. However, due to circumstances beyond control, only four dams on the LA-rich diet had successful birthing outcomes; this is reflected in the sometimes higher SEMs of data. de Groot *et al.* [240] found no effect of 10.9 g LA supplementation compared to combined supplementation of 2.8 g ALA and 9.0 g LA on birthing outcomes of women, suggesting that successful birthing outcomes may not have been affected by high intake of LA or ALA.

There were some variations of body weight between dietary groups at different stages of the trial, but not at day 21 (day of weaning) or day 78 (day of euthanisation). Variation at different days in the trial is in agreement with the findings of 2.3.2 and 3.3.1, and an increase in body weight is in agreement with the results of Poiley [157].

At euthanisation, brains of animals receiving the control diet were lighter than those on the experimental diets. This is as was expected, as the experimental diets were higher fat (10 %) than the control diet (3 %), and the brain has a high fat content. Mice undergo a period of rapid brain growth in the first 3 to 4 weeks of life [241], the period in which animals were receiving experimental and control diets. In previous chapters, experimental diets were consumed from 3 to 4 weeks of age. As such, dietary fatty acids may have more effect on brain weight during periods of rapid growth. Whether this increase in brain size had an influence on functional outcomes like learning behaviour etc, was beyond the scope

of this experiment. There was no effect of diet on spleen, thymus, liver, or epididymal fat weight, in accordance with findings from previous chapters.

5.4.4. Airway Responsiveness

There was no effect of diet on airway responsiveness to methacholine at any of the concentrations measured. This is in agreement with the findings of 3.3.4. In humans, links between dietary fat in pregnancy and atopic asthma in children have been suggested, but are often complicated by the nature of the design of the trial involved. Many of these studies tend to focus on the effect of the long chain *n-3* PUFAs rather than their precursors. Retrospective studies rely on dietary recall, often several years later on, which may not be an accurate reflection of actual dietary intake [242], whilst cohort studies in humans may not allow examination of effect of diet on particular time periods. Crozier *et al.* [236] found no change in fruit and vegetable consumption during and after pregnancy; whilst this study did not examine all sources of ALA and LA, it does suggest that diet may not change during and after pregnancy, and as such, cohort studies may not be able to identify the effect of dietary fatty acids at a particular critical window of time. A more accurate human study would be an intervention study, but financial constraints can make them impractical, and as such human intervention studies examining the effect of LA and ALA in pregnancy on asthma of offspring has not yet been carried out. A prospective cohort study, carried out by Miyake *et al.* [243] showed a negative relationship between ALA intake in pregnancy and infantile wheeze (but not eczema), and a positive relationship between LA in pregnancy and eczema (but not wheeze) at 16 to 24 months of age.

The fatty acid composition of cord blood is a useful indicator of foetal fatty acid status, and is affected by maternal dietary intake, placental transport, and maternal, placental and foetal metabolism [230]. Newson *et al.* [244] demonstrated a positive relationship between ratio of LA to ALA in cord blood, and onset of wheeze at 30 to 42 months of age (there was no relationship with wheeze at six months of age). As such, it is possible that there may be an effect seen in later life that is not observed earlier in life; there may have been an increase in asthma in mice observed if airway responsiveness of mice had been tested at a different age. Dirix *et al.* [245], however, found no effect of AA content of umbilical cord plasma, and arterial and venous wall phospholipids, and lung function of children at 7 years of age. Whether a relationship would have been found if AA content of alternative lipid fractions (i.e. TAG etc) or lipid sources (i.e. erythrocytes etc) had been assessed remains to be determined.

5.4.5. Acute Skin Test

Ear swelling in response to ovalbumin was lower in animals whose dams had received the ALA-rich in gestation and / or lactation, and those receiving the LA-rich diet in gestation compared to those receiving the low fat control diet for the whole period. Animals in the AL group had a lower ear swelling response than the LGL and LL groups. Results from 3.3.5 suggested that there was no difference between ear swelling in animals receiving the ALA-rich linseed oil diet or the LA-rich sunflower oil diet. The apparent disparity between results from this and the current study may stem from two main reasons. Firstly, different diets were used – the current study used synthetic diets, formulated to be rich in either LA or ALA, whilst the study in 0 used linseed or sunflower oil as the fat source, which would, therefore, contain a different background of fatty acid composition. Secondly, there may be an effect of timing of fatty acid exposure. This is suggested by the findings of the current study, where animals receiving ALA-rich diet in lactation had a reduced ear swelling compared to those receiving the LA-rich diet in both gestation and lactation, or lactation alone. This apparent effect of time could be due to the nutritional insult occurring at a critical window of development, or simply because the fatty acids were consumed recently enough to have an effect. Studies to date on immediate hypersensitivity and fatty acid intake in pregnancy have tended to focus on the effect of fish oil, rather than the short chain *n-3* or *n-6* PUFAs, and as such, it is difficult to ascertain whether ALA and LA do have a critical window of effect in pregnancy on immediate hypersensitivity.

5.4.6. Antibody Production

There was no effect of diet in gestation or lactation on IgE, IgG1 or IgG2a production. T_H1 cells produce IFN-γ, which causes antibody isotype switching to IgG2a. T_H2 cells, however, produce IL-4, which causes isotype switching to IgE, which is the antibody responsible for immediate hypersensitivity and promotes IgG1 production. These results, therefore, suggest that LA and ALA in gestation and / or lactation may not have a physiological effect on immediate hypersensitivity response in ovalbumin-sensitised mice as indicated by level of antibody production. As with previous discussions in this chapter, studies carried out to date have either focussed on the effect of LA or ALA outside of pregnancy, or have examined the effect of fish or fish oil in pregnancy on antibody production. There were no differences found in IgE, IgG1 or IgG2 levels in plasma between the ALA-rich linseed oil or LA-rich sunflower oil dietary groups. It is possible, however, that sensitisation may not have been effective, which would explain the reason

for the 10-fold reduction in IgE production in the current study compared the previous allergic response study (3.3.6).

5.4.7. Cytokine Analysis

There was no effect of diet in gestation or lactation on IL-5 content of BALF, or IL-4, IL-10, IL-5 or IFN-γ production by cultured spleen cells in response to anti-CD3 or OVA. IL-4, IL-5 and IL-10 are produced by activated T_H2 cells; IL-4 causes naïve T-cells to differentiate into T_H2 cells, IL-5 increases production and activation of eosinophils, whilst IL-10 acts to inhibit activation and growth of T_H1 cells. IL-4 and IL-5 are also produced by activated mast cells. IFN-γ is produced by NK cells, T_H1 cells and CTLs; it is the signature cytokine of T_H1 cells, in much the same way that IL-4 is the signature cytokine of T_H2 cells. IFN-γ activates macrophages, causes isotype switching to opsonising and complement-fixing antibodies, and promotes differentiation of naïve T-cells to T_H1 cells, whilst inhibiting proliferation of T_H2 cells. However, the reduction in level of cytokines produced in response to stimulant, compared to 3.3.7 suggests that sensitisation may not have been successful.

Previous studies have either examined long chain *n-3* PUFAs in pregnancy or short-chain PUFAs in non-pregnant beings. In 3.3.7 there was no difference found between cytokine production between the (LA-rich) sunflower oil and (ALA-rich) linseed oil dietary groups. However, this chapter did not examine the effect of pregnancy, and since the current study did not appear to have successfully sensitised animals means that the effect of these fatty acids in pregnancy on allergic response in offspring is still unclear.

5.4.8. Histological Analysis

Cortex / medulla ratio was examined because as previous research at the University of Utrecht suggested that DHA may reduce medulla regions in the thymus [246 unpublished work], and to allow continuum with other chapters of research carried out at the University of Utrecht. T-cells mature in the thymus. The cortex contains the immature T-cells, which migrate to the medulla as they mature. Therefore, any effect on ratio of cortex to medulla may alter the area available for T-cell maturation or may indicate an effect on maturation. In agreement with findings from previous chapters (2.3.8 and 3.3.10), there was no effect of diet on cortex / medulla ratio of thymuses.

5.4.9. Immune Cell Subset Analysis

There was no effect of diet in gestation or lactation on abundance of eosinophils, neutrophils or macrophages in BALF, or cell surface expression markers in bone marrow. This is in agreement with findings from previous chapters and 1.4. However, in discordance with them, there were differences in expression of cell surface markers in the thymus, and spleen. CD3+ expression in thymus cells was lowest in the control and AG groups, and higher in the LG, LGL and LL groups than in the AL group. CD3+ cells that also expressed CD4+ cells were lowest in the LG and LGL groups, and highest in the AGL and LL groups. CD3+ cells that also expressed CD8+ in the thymus were lower in the control, AG, AGL and AL groups than in the LG and LGL groups. There was no effect of diet on CD3+CD4+ cells that were also CD69+. Expression of CD62L+ on CD3+CD4+ thymus cells was higher in the control and AG groups than all other groups. CD4+CD25+ expression in thymus cells was higher in the AG and LL groups than the AGL and AL groups. Expression of FoxP3+ in the CD4+CD25+ thymus cells was lowest in the control group, and highest in the LG, LGL and LL groups. F4-80 expression in the spleen was highest in the AL group, and lowest in the control, AG, LG and LL groups. There was no difference in CD3+ expression between dietary groups. CD3+ cells that also expressed CD4+ abundance was highest in the AGL, AL and LL groups; these groups also had the lowest expression of CD8+. CD3+CD4+ cells that also expressed CD62L+ abundance was lower in the LG and LGL groups than the LL group. CD3+CD4+ cells that were CD69+ were lowest in the LL group, and higher in the control group than the AGL and LGL groups. CD19+ expression was lower in the AGL, AL and LL groups than the control and 1G groups. CD4+CD25+ expression was lower in the AL group than the AGL, LGL and LL groups, and lower in the LGL group than the LL group. FoxP3+ expression in CD4+CD25+ cells was lower in the control and AG groups than the AGL, AL, and LL groups. CD94+ expression was lower in the control group than in the LL group. Statistical calculations on insufficient sample sizes do not account for these differences. However, some dietary groups have large SEMs, which raises questions as to the accuracy of the data.

5.4.10. Limitations

Due to the unpredictable nature of number of offspring in breeding trials, some groups had small sample sizes (AGL and LGL). This limits the usefulness of data as *n*-numbers of some groups are too small to be statistically accurate; this is reflected in the large SEMs seen in data summaries. In addition, the reduction in IgE and cytokine

production, compared to results from a previous chapter suggests that sensitisation may not have been successful. Time constraints have prevented the study from being repeated, and as such, differences seen in these groups should not be relied upon. They are included for completeness of the study.

This study used male offspring; due to the gender differences in metabolism of short chain PUFAs to long chain PUFAs [201], differences may be observed in female offspring. This effect has been observed in humans, but not examined in mouse models. In addition, since the current study was carried out in mice, it may be questioned as to whether similar effects would be observed in humans

5.4.11. Conclusions

This study set out to test hypotheses of reduced severity with ALA-rich diet in pregnancy / pregnancy and lactation and increased severity with LA-rich diet in pregnancy / pregnancy and lactation. It was hypothesised that increased ALA or LA intake in lactation would have less of an effect than increased ALA or LA intake in pregnancy. Using ear swelling in response to ovalbumin, to which the mice had been sensitised, as the outcome, animals whose dams had received the ALA-rich in gestation and lactation, and those receiving the LA-rich diet in gestation had lower ear swelling compared to those receiving the low fat control diet for the whole period. Animals receiving the ALA-rich diet in lactation had lower ear swelling response than those receiving the LA-rich diet in gestation and lactation, or lactation alone. Despite these differences, there were no effects on lung function or immune markers measured in BALF, plasma, thymocytes, bone marrow or splenocytes. In addition, it does not appear that sensitisation was completely successful.

Studies examining the effect of fatty acids in pregnancy to date have focussed on the long chain *n-3* PUFAs. Studies examining the effect of the shorter chain fatty acids have instead focussed on non-pregnant beings. If there is a critical window of effect of the short chain PUFAs on allergic sensitisation, these studies may have been carried out at too late a stage of development for an effect to be observed. Problems with sample size have prevented, in certain groups, statistically reliable data from being produced, which is reflected in the large error ranges seen. Data from these groups are included in order to provide completeness of the study, but should not be reported as statistically accurate. The ideal situation would be to repeat the study, but financial and time constraints have prevented this from happening to date.

Chapter 6. Effect of a Low Protein Diet in Pregnancy on the Immune System of Offspring in Two Generations

6.1. Introduction

The previous chapters suggested that diet in pregnancy may affect the immune system of offspring. However, the effect of diet in pregnancy on the immune system of offspring in more than one generation has not been investigated to date. A rat model of protein restriction was employed as it has much-used in the department, and the effect of protein restriction on the physiology of the offspring has been well-established [247].

6.1.1. Low Protein Model

The low protein model in pregnant rats (9% dietary protein vs. 18% in the control) has been shown to produce offspring with low birth weight that are hypertensive at birth and in later life [248]. Protein restriction reduces 11β-hydroxysteroid dehydrogenase in the placenta and therefore increases foetal exposure to glucocorticoids, thereby inducing hypertension in the offspring [248, 249]. Protein restriction in pregnancy and the resulting maternal stress therefore could affect the development of any organ system in the foetus, including the immune system. Experiments using this model have examined physiological and genetic effects of the maternal protein restriction, such as effects on hypertension [248], kidney function [250], vascular function [251], glucose tolerance [252], gene regulation [139, 253, 254] and bone mass [255] in the offspring. More recently, an effect on the second generation of diet from the F0 generation has been discovered (see 6.1.3) [139]. There are no systematic studies of the effect of this maternal protein restriction on immune function in the offspring in the next or subsequent generations.

6.1.2. Effect of Pre-natal Stress on the Immune System

Pre-natal stress can be induced by a number of different methods, and whilst the effect of protein restriction in pregnancy on the immune system has not been looked at directly, the effect of other types of pre-natal stress on the immune system have been. In rats, environmental stress in pregnancy has been shown to reduce T- and B-cell proliferation in offspring [136], and levels of IgG in serum [137], whilst social stress has been shown to reduce abundance of T_H cells in the offspring [138].

6.1.3. Effects of Diet in Pregnancy on Later Generations

Between November 1944 and May 1945, a severe rationing was imposed on the Netherlands by the Nazis, and as a consequence, calorific intake was reduced from about

1800 to 400 – 800 calories per day [129], in a period named the Dutch Hunger Winter. On liberation of the Netherlands by the Allied forces, calorific intake returned to adequate levels almost immediately. It therefore provides a means of analysis of the effect of famine on a population with women in early, mid and late trimesters of pregnancy, who were used to sufficient nutritional intake. By following offspring of these women in later life, insufficient nutrient availability *in utero* has been linked to effects in the next generation, such as increased mortality [256], reduced birth weight [257], increased adiposity and poor health in later life [258]. Using the same rat model as will be used in the study described in this chapter, reduced protein intake in the F0 generation has been shown to alter the methylation of the PPAR-α and glucocorticoid receptor genes in both the F1 and F2 generations [139], suggesting intergenerational effects of maternal diet. However, the effect of altered maternal diet in pregnancy across more than one generation is a relatively new area of research, and the effect on immunological status of the F2 generation has not been examined.

6.1.4. Aims of the Study

This study aimed to determine the effect on the immune system over two generations of diets consumed during rat pregnancy that provided adequate or low protein. Altering maternal F0 protein intake is known to induce a stress response [247-249, 259] that may impact on foetal immune development, in particular T-, T_H and B-cell abundance. Furthermore the low maternal protein diet may cause structural changes in foetal and/or maternal immune cells, which may affect immune function of the F0 and F1 generation. For example, offspring of mothers fed the low protein diet were demonstrated to have altered phospholipid fatty acid compositions in the plasma, liver, heart, lung and brain at weaning [260]. It is highly likely that such effects occurred within the immune system as well. The effects of early protein restriction on immune function (if they occur) may be passed to the F2 generation. The study aims to determine whether maternal protein restriction alters immune function in the F1 generation and whether such a change, if it occurs, is maintained in the later generation (i.e. F2). The study is summarised in Figure 6-1.

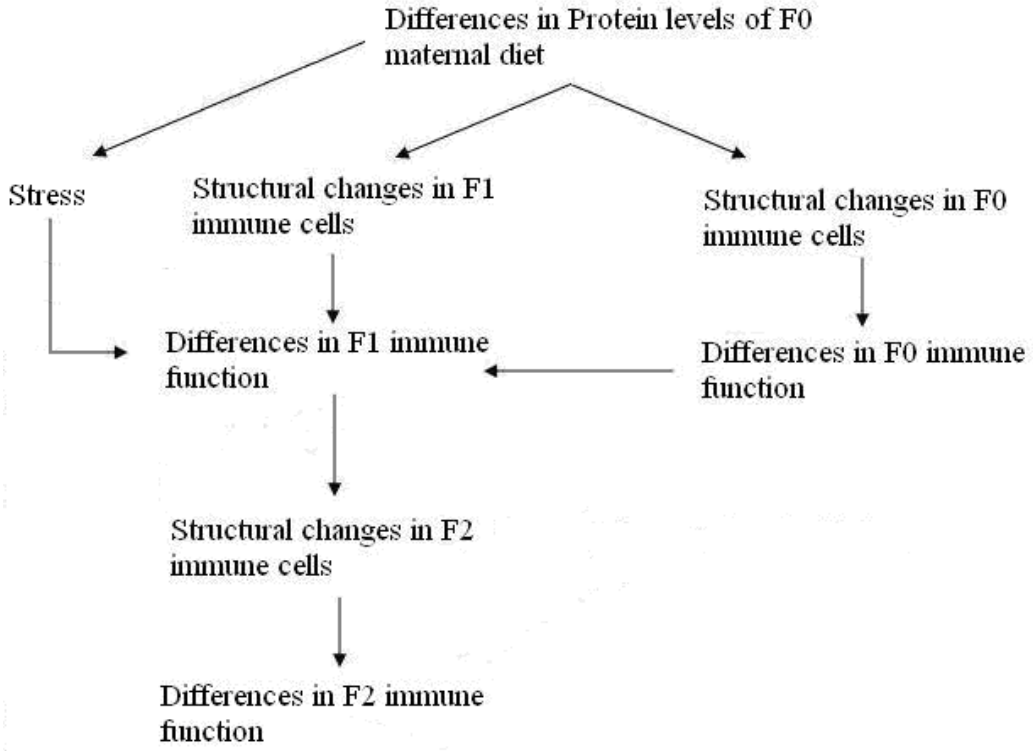

Figure 6-1: Causal chain linking altered maternal protein intake to altered offspring immune function. Changes in protein composition of the F0 maternal diet may cause changes in structure of foetal (F1) and / or maternal (F0) immune cells, or induce stress, all of which may in turn affect the function of F1 immune cells. This may cause structural and / or functional changes in the F2 generation.

In order to achieve this aim, three diets were fed to rats in gestation over two generations, resulting in six dietary groups. This is summarised in Table 5-1. Additional folate was added to the *in utero* diets of the F2 generation, as folate has been shown to reverse the programming effects of a low protein diet [129, 261].

Table 6-1: Diets used in this trial.

Diet name	F0 Diet	F1 Diet
C	Control	-
PR	Protein Restricted	-
C-C	Control	Control
C-CF	Control	Control + Folate
PR-C	Protein Restricted	Control
PR-CF	Protein Restricted	Control + Folate

6.1.5. Hypothesis

This study set out to test the following hypotheses:

- The PR and PR-C groups will have a lower lymphoid tissue weights than other dietary groups
- T_H cell and B-cell abundance will be reduced in PR offspring.
- Cytokine profiles representing T_H1 and T_H2 skewing will be altered in PR offspring.
- Any changes observed in the F1 generation, will be seen in the F2 generation.

6.2. Materials and Methods

6.2.1. Animals, Diets and Materials

6.2.1.1. Animals

Male and female Wistar rats were obtained from the Biomedical Research Facility, University of Southampton (Southampton, UK). Following mating, dams were housed in groups of six for the first 14 days of pregnancy, and individually from day 14 of pregnancy to birth, in shoebox cages. Animals were kept at 22°C ± 2°C, under a 12 hour light: 12 hour dark cycle in temperature and light-controlled room in the animal facility at the University of Southampton. Offspring remained with dams until day 28, when they were euthanized or moved into group cages until day 120. Animals had *ad libitum* access to tap water and food. All studies were carried out in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, under project licence number 70/685, and personal license number 70/20024.

6.2.1.2. Diets

F0 animals received a control (C; 18 % by weight protein) or protein restricted (PR; 9 % by weight protein) diet during pregnancy. In the PR diet, protein was replaced by carbohydrate. C and PR diets were isocaloric. F1 animals received a control + folate (CF), or a control diet (without additional folate, same as the control diet used in the F0 pregnancy) during pregnancy. The control and protein restricted diets contained 1 mg / kg folate. The control + folate diet contained 5 mg / kg folate. The increase in folate provided by this diet is comparable to the UK recommendation of folate intake for women in the first trimester of pregnancy [261]. During lactation, all animals received a lactation diet (AI*N*-*93*G). Animals were weaned onto a maintenance diet (AI*N*-*93*M). Diets were obtained from Test Diet (Richmond, IN, USA). Macronutrient composition of diets is shown in Table 6-2.

Table 6-2: Macronutrient composition of control (C), control + folate (CF), protein restricted (PR), AI*N*-*93*G (lactation diet), and AI*N*-*93*M (maintenance diet) diets. Taken from Test Diet [262-266]

Product	C	CF	PR	AI*N*-*93*G	AI*N*-*93*M
Carbohydrate (g / 100 g)	63.9	63.9	73.0	63.9	73.0
Protein (g / 100 g)	16.9	16.9	8.9	18.1	13.0
Fat (g / 100 g)	10.1	10.1	10.1	7.1	4.1
Fibre (g / 100g)	5.0	5.0	5.0	5.0	5.0
Mineral Mix (mg / 100 g)	20.0	20.0	20.0	35.0	35.0
Vitamin Mix (mg / 100 g)	5.0	5.0	5.0	10	10.0
Folate	1.1	5.1	1.1	2.0	2.1

Diets were analysed for fatty acid content using the method described previously (2.2.2.3); fatty acid composition of diets is shown in Appendix Five and fatty acids of interest in Figure 6-2. There were no major differences in the fatty acid compositions of the different diets.

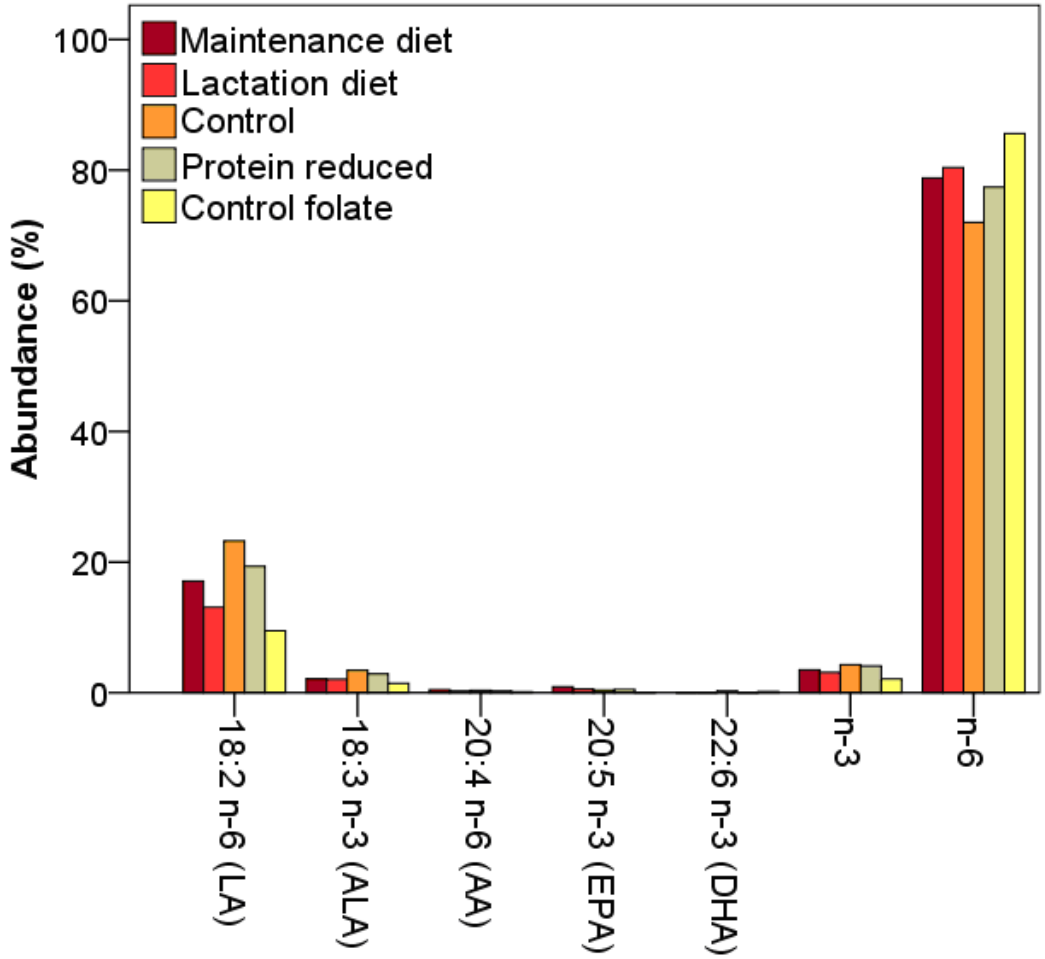

Figure 6-2: Profile of individual and total *n-3* and *n-6* PUFAs in the diets used

6.2.1.3. Materials

Materials used were sourced from the same origins as stated previously (2.2.1.3). In addition, ELISA plates were bought from Invitrogen (Paisley, UK).

6.2.2. Methods

An overview of the trial is provided in Figure 2-4. Fourteen virgin 120 day old Wistar rats (F0) were mated and fed either a control (18 % protein) or protein-reduced (9 % protein) diet; both diets contained 10.1 % fat with the source being soybean oil. Litters were reduced to 8, within 24 hours, maximising for females. During lactation, dams received a lactation diet (AI*N*-*93*G). Offspring (F1) were weaned onto a maintenance diet (AI*N*-*93*M), which contained 4.1 % fat, with the source being soybean oil. When F1 males were 28 days old, and females were 120 days old, animals were euthanized and tissues harvested. F1 females were mated before sacrifice and animals that had received the control diet in the F1 pregnancies were transferred to the same diet, or a control plus folate diet (same level and source of fat as for the F0 diets). The control + folate diet contained 5 mg / kg folate; the control diet contained 1 mg / kg folate. Animals that had received the protein reduced diet were transferred to control diets with or without folate (same level and source of fat as for the F0 diets). Additional folate was added to these diets because it reverses the effects of protein restriction [261]. Lactating dams received the lactation diet (AI*N*-*93*G). Offspring (F2) were transferred to the maintenance diet (AI*N*-*93*M). F2 males were euthanized at 28 days, and females at 120 days. F2 females had also been mated prior to euthanisation (although samples of F3 offspring were not collected for this trial). Animals were euthanized using the method described in 4.2.2. From all sampling points, spleens and thymuses were collected and flow cytometry carried out to determine cell types present. For day 120 females, cells were cultured for lymphocyte proliferation and cytokine analysis. Supernatants from cell cultures were analysed for IFN-γ and IL-4 using ELISAs. It was not possible to do these measurements for day 28 males, as insufficient cells were available. Animals were housed separately in shoebox cages, and kept at 21 ± 2 °C, under a 12 hour light : 12 hour dark cycle. At all times, animals had *ad libitum* access to food and water. All studies were carried out in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, under project licence number 30/1889, and personal license number 70/20024.

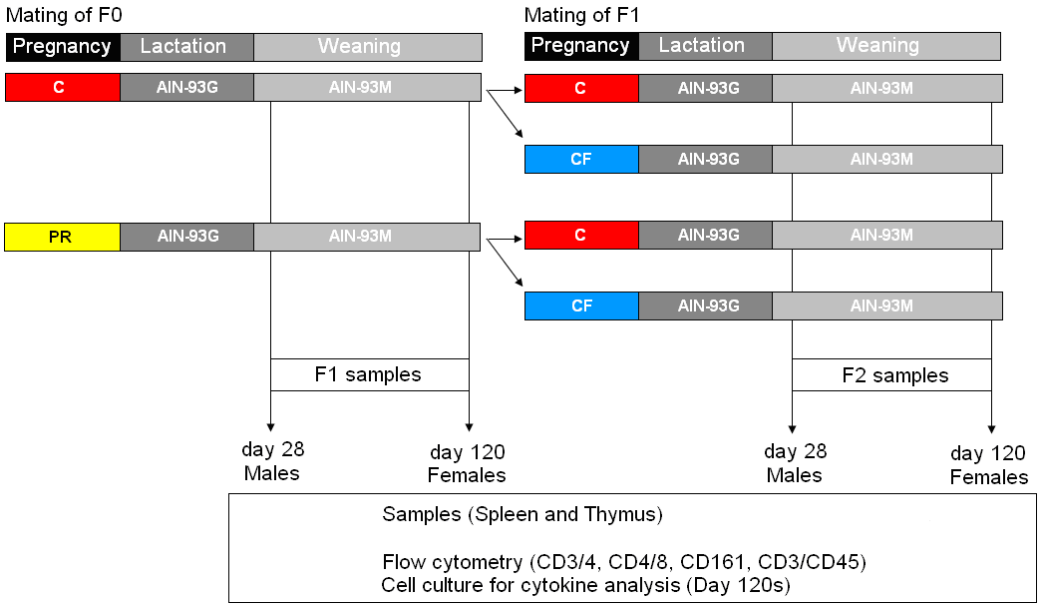

Figure 6-3: Schematic diagram of multi-generational low protein study. C, Control diet; CF, Control + Folate diet; PR, Protein Reduced Diet.

6.2.2.1. Preparation of Cells from Lymphoid Tissues

Females aged 120 days, and males aged 28 days were euthanized and cells prepared for cell counting, flow cytometry, and cytokine production as described in 4.2.2.2.

6.2.2.2. Determination of Cell Numbers in Lymphoid Tissues

Cells of spleen and thymus tissues were counted using the method described in 4.2.2.3.

6.2.2.3. Determination of Cell Subsets in Lymphoid Tissues using Flow Cytometry

Cells underwent flow cytometry (FACs) analysis using the method and antibody markers described in 4.2.2.4.

6.2.2.4. Determination of Cytokine Production by Spleen and Thymus Cells

1 ml of a 5 x 10^6 cells / ml suspension of spleen or thymus cells was put in wells of a 12-well flat-bottom plate, and 100 µl foetal calf serum, 700 µl RPMI and 200 µl Con A (at concentrations 0, 2.5, 5, 10 or 20 µg/ml) added to each well. Plates were incubated at 37°C in 5% CO_2 for 48 h, and then centrifuged at 1000 rpm for five minutes, with a low

brake. Cell culture supernatants were placed in eppendorfs, and stored at –20°C until analysis. Cell supernatants were analysed for IL-4 and IFN-γ content following the manufacturer's instructions.

6.2.2.5. Statistical Analysis

Results were analysed using SPSS version 15.0. Parameters were analysed using Levene's test of homogeneity to ensure groups had equal variances, in which case they were compared using one-way analysis of variance (ANOVA), followed by *post-hoc* testing (Tukey). If Levene's test showed unequal variances to be present, groups were compared using the Kruskal-Wallis test. Where this was used, it is reported in the results section. If the Kruskal-Wallis test indicated a significant effect, Mann-Whitney U independent group comparisons tests were then carried out to compare each combination of groups.

6.3. Results

For all results, "dietary group of X generation" refers to the diet received whilst *in utero*, i.e. the diet of X's mother during pregnancy.

6.3.1. Gestational Data for Rats fed the Different Diets in Pregnancy

Effect of diet on duration of pregnancy, litter size, number of males and females, and the ratio of males to females in the litter is shown in Table 6-3. There was no effect of diet on any of these parameters measured at either the F1 or F2 generations.

Table 6-3: Effect of diet on duration of pregnancy, litter size, number of males and females, and ratio of males to females as a result of diet in pregnancy over two generations. Ratio of males to females in the F1 generation were analysed using non-parametric testing.

	Dietary group of F1 generation			
	C (n = 12)		PR (n = 11)	
Duration of pregnancy (days)	22.0 ± 0.2		22.4 ± 0.2	
Litter size (n)	11.6 ± 0.6		12.7 ± 0.8	
Males (n)	5.8 ± 0.6		6.3 ± 0.6	
Females (n)	5.8 ± 0.8		6.3 ± 0.5	
Ratio of males to females	1.4 ± 0.3		1.0 ± 0.1	
	Dietary group of F2 generation			
	C-C (n = 10)	**C-CF (n = 11)**	**PR-C (n = 10)**	**PR-CF (n = 8)**
Duration of pregnancy (days)	21.9 ± 0.4	21.9 ± 0.2	22.8 ± 1.1	22.1 ± 0.4
Litter size (n)	12.2 ± 0.6	12.5 ± 0.8	12.3 ± 0.6	10.9 ± 0.9
Males (n)	6.3 ± 0.5	6.4 ± 0.8	6.2 ± 0.6	5.1 ± 0.7
Females (n)	5.9 ± 0.6	6.1 ± 0.5	6.1 ± 0.6	5.8 ± 0.6
Ratio of males to females	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2

6.3.2. Lymphoid Tissue Weights of Offspring of Rats fed the Different Diets in Pregnancy

Data on effect of diet in gestation on thymus, and spleen weights in F1 and F2 generations are shown in Table 6-4 (day 28 males) and Table 6-5 (day 120 females). There was no effect of thymus and spleen weight in the F1 or F2 males, or the F1 females. However, in the F2 females (120 days old), thymus weight was greater in the C-C group compared to the C-CF and PR-C groups, and spleen weight was greater in the C-C group compared to the PR-CF group.

Table 6-4: Thymus and spleen weights of two generations of young (28 days old) male rats receiving experimental diets *in utero*.

	Dietary group of F1 generation			
	C (n = 23)		PR (n = 13)	
Thymus weight (g)	0.37 ± 0.01		0.40 ± 0.01	
Spleen weight (g)	0.35 ± 0.01		0.33 ± 0.01	
	Dietary group of F2 generation			
	C-C (n = 7)	C-CF (n = 7)	PR-C (n = 5)	PR-CF (n = 4)
Thymus weight (g)	0.37 ± 0.03	0.38 ± 0.02	0.45 ± 0.03	0.39 ± 0.02
Spleen weight (g)	0.40 ± 0.04	0.35 ± 0.03	0.38 ± 0.04	0.32 ± 0.01

Table 6-5: Thymus and spleen weights of two generations of adult (120 days old) female rats receiving experimental diets *in utero*. Values with different letters denote differences between dietary groups.

	Dietary group of F1 generation			
	C (n = 7)		PR (n = 12)	
Thymus weight (g)	0.60 ± 0.05		0.62 ± 0.03	
Spleen weight (g)	0.67 ± 0.03		0.68 ± 0.02	
	Dietary group of F2 generation			
	C-C (n = 7)	C-CF (n = 11)	PR-C (n = 7)	PR-CF (n = 9)
Thymus weight (g)	0.77 ± 0.03^{b}	0.64 ± 0.03^{a}	0.63 ± 0.04^{a}	0.69 ± 0.03^{ab}
Spleen weight (g)	0.76 ± 0.05^{b}	0.70 ± 0.03^{ab}	0.66 ± 0.02^{ab}	0.61 ± 0.03^{a}

6.3.3. Cell Counts of Lymphoid Tissues of Offspring of Rats receiving the Different Diets in Pregnancy

Data on effect of diet on cell counts of thymus and spleens are shown in Table 6-6 and

Table 6-7. There was no difference in cell counts of spleens and thymuses in the F1 generation males. In the F2 generation, cell counts were highest in the PR-C generation than the C-C and C-CF diets for both spleens and thymuses. However, the large SEM suggests this may be an artefact of sample size. There was no difference between PR-C and PR-CF groups for absolute number of spleen cells, absolute or total number of cells in the thymus, but PR-C group had a higher total number of cells in the spleen than the PR-CF group. There was no difference in cell count between dietary groups in either the F1 or F2 generations of the females.

Table 6-6: Cell counts of thymus and spleens from two generations of young (28 days old) male rats receiving experimental diets the F2 generation was analysed using non-parametric testing. Values with different letters denote differences betwee

	Dietary group of F1 generation	
	C (n = 23)	**PR (n = 13)**
Number of cells per mg of spleen (x 10^4)	40.91 ± 4.12	41.15 ± 5.66
Absolute number of spleen cells (x 10^6)	14.06 ± 1.27	13.28 ± 1.64
Number of cells per mg of thymus (x 10^4)	82.07 ± 24.99	97.48 ± 29.10
Absolute number of thymus cells (x 10^6)	31.27± 10.46	42.26 ± 15.04

	Dietary group of F2 generation			
	C-C (n = 7)	**C-CF (n = 7)**	**PR-C (n = 5)**	**PR- (n =**
Number of cells per mg of spleen (x 10^4)	34.76 ± 11.29[a]	41.95 ± 7.73[a]	113.14 ± 7.31[b]	67.08 ±
Absolute number of spleen cells (x 10^6)	15.92 ± 7.44[a]	14.51 ± 2.75[a]	41.50 ± 2.23[b]	21.32 ±
Number of cells per mg of thymus (x 10^4)	13.35 ± 3.54[a]	19.72 ± 5.25[a]	109.17 ± 36.17[b]	33.81 ±
Absolute number of thymus cells (x 10^6)	5.15 ± 1.61[a]	7.81 ± 2.47[a]	51.89 ± 19.48[b]	13.62 ±

Table 6-7: Cell counts of thymus and spleens from two generations of adult (120 days old) female rats receiving experimental diet of diet on any of the parameters measured.

	Dietary group of F1 generation	
	C (n = 7)	**PR (n = 12)**
Number of cells per mg of spleen (x 10^4)	31.16 ± 4.25	31.01 ± 5.66
Absolute number of spleen cells (x 10^6)	21.05 ± 3.71	21.70 ± 4.19
Number of cells per mg of thymus (x 10^4)	21.17 ± 2.59	27.38 ± 3.71
Absolute number of thymus cells (x 10^6)	12.70 ± 1.98	16.78 ± 2.16

	Dietary group of F2 generation			
	C-C (n = 7)	**C-CF (n = 11)**	**PR-C (n = 7)**	**PR-C (n = 9**
Number of cells per mg of spleen (x 10^4)	45.22 ± 8.13	60.53 ± 14.06	35.46 ± 7.92	37.12 ± 8
Absolute number of spleen cells (x 10^6)	32.24 ± 4.65	42.87 ± 11.22	23.68 ± 5.39	23.41 ± 6
Number of cells per mg of thymus (x 10^4)	52.98 ± 11.58	62.33 ± 22.71	55.44 ± 9.81	35.52 ± 5
Absolute number of thymus cells (x 10^6)	40.98 ± 8.90	43.35 ± 19.27	34.21 ± 6.29	24.36 ± 3

6.3.4. Cell Subsets in the Thymus, Spleen, Blood and Bone Marrow of Offspring of Rats fed the Different Diets in Pregnancy

Abundance of cell subsets in the spleen of young rats (Table 6-8), adult rats (Table 6-9), and thymuses of young (Table 6-10) and adult rats (Table 6-11) are shown below. There were no differences in any of the parameters measured, except for the F2 generation males, where T_H cell abundance was higher in C-CF group than the PR-C and PR-CF groups, and B-cell abundance was higher in the PR-C group than the C-C and C-CF groups.

Table 6-8: Abundance of subsets of splenic lymphocytes of two generations of young (28 days old) male rats receiving experimental diets in utero. Results are shown as mean ± SEM. Abundance is shown as % of splenocytes.

	Dietary group of F1 generation			
	C (n=22)		PR (n=13)	
CTLs	11.40 ± 0.63		10.33 ± 0.88	
T_H cells	17.75 ± 1.54		17.52 ± 1.90	
B cells	43.51 ± 3.00		46.46 ± 4.98	
NK cells	2.52 ± 0.16		2.67 ± 0.36	
Splenic Macrophages	4.68 ± 0.50		4.62 ± 0.58	
	Dietary group of F2 generation			
	C-C (n = 7)	C-CF (n = 7)	PR-C (n = 5)	PR-CF (n = 4)
CTLs	14.87 ± 1.81	16.39 ± 0.92	14.19 ± 1.94	19.01 ± 2.99
T_H cells	12.80 ± 2.14^{ab}	18.49 ± 2.18^{b}	7.74 ± 0.46^{a}	9.47 ± 0.26^{a}
B cells	47.31 ± 3.35^{a}	44.21 ± 2.07^{a}	60.36 ± 2.23^{b}	49.37 ± 2.94^{ab}
NK cells	5.37 ± 0.73	4.15 ± 0.81	2.76 ± 0.40	5.06 ± 1.99
Splenic Macrophages	2.48 ± 0.43	3.57 ± 0.91	2.72 ± 0.94	4.62 ± 0.79

Table 6-9: Abundance of subsets of splenic lymphocytes of two generations of adult (120 days old) female rats receiving experimental diets *in utero*. Results are shown as mean ± SEM. Abundance is shown as % of splenocytes.

	Dietary group of F1 generation			
	C (n = 7)		PR (n = 12)	
CTLs	28.15 ± 3.82		21.58 ± 4.55	
T_H cells	7.22 ± 1.36		12.71 ± 3.85	
B cells	40.86 ± 3.71		38.83 ± 6.75	
NK cells	9.30 ± 2.11		8.41 ± 2.58	
Splenic Macrophages	2.13 ± 0.46		1.96 ± 0.37	
	Dietary group of F2 generation			
	C-C (n = 7)	C-CF (n = 11)	PR-C (n = 7)	PR-CF (n = 9)
CTLs	29.04 ± 2.11	35.49 ± 3.39	31.59 ± 1.18	27.02 ± 1.11
T_H cells	4.20 ± 1.04	7.01 ± 0.75	5.82 ± 0.62	7.07 ± 0.98
B cells	33.58 ± 5.76	33.63 ± 3.46	39.57 ± 3.23	40.72 ± 4.42
NK cells	11.84 ± 4.74	7.64 ± 1.94	12.29 ± 2.18	14.06 ± 2.66
Splenic Macrophages	1.51 ± 0.20	1.83 ± 0.31	2.72 ± 0.42	2.90 ± 0.50

Table 6-10: Abundance of subsets of thymic lymphocytes of two generations of young (28 days old) male rats receiving experimental diets in utero. Results are shown as mean ± SEM. Abundance is shown as % of thymocytes.

	Dietary group of F1 generation			
	C (n=22)		PR (n=13)	
CTLs	8.58 ± 0.66		11.03 ± 1.06	
T_H cells	19.33 ± 1.60		23.97 ± 2.09	
B cells	0.42 ± 0.06		0.70 ± 0.17	
NK cells	0.27 ± 0.04		0.29 ± 0.03	
	Dietary group of F2 generation			
	C-C (n = 7)	C-CF (n = 7)	PR-C (n = 5)	PR-CF (n = 4)
CTLs	13.34 ± 4.36	12.57 ± 4.78	34.53 ± 5.03	24.79 ± 9.40
T_H cells	6.87 ± 0.54	8.84 ± 1.43	13.60 ± 1.02	14.42 ± 1.54
B cells	0.54 ± 0.16	0.38 ± 0.07	0.86 ± 0.25	0.89 ± 0.19
NK cells	0.17 ± 0.09	0.08 ± 0.01	0.33 ± 0.20	0.21 ± 0.04

Table 6-11: Abundance of subsets of thymic lymphocytes of two generations of adult (120 days old) female rats receiving experimental diets in utero. Results are shown as mean ± SEM. Abundance is shown as % of thymocytes.

	Dietary group of F1 generation	
	C (n = 7)	PR (n = 12)
CTLs	37.13 ± 4.15	24.16 ± 4.85
T_H cells	20.00 ± 2.25	22.45 ± 4.42
B cells	5.33 ± 0.60	10.35 ± 6.11
NK cells	1.12 ± 0.10	1.35 ± 0.40

	Dietary group of F2 generation			
	C-C (n = 7)	C-CF (n = 11)	PR-C (n = 7)	PR-CF (n = 9)
CTLs	42.02 ± 3.43	49.09 ± 2.27	38.12 ± 2.16	39.01 ± 3.27
T_H cells	15.13 ± 2.66	19.59 ± 1.41	15.33 ± 1.35	14.47 ± 2.37
B cells	4.35 ± 1.06	3.98 ± 0.84	3.28 ± 0.42	3.84 ± 1.33
NK cells	0.93 ± 0.13	0.97 ± 0.13	0.76 ± 0.12	0.82 ± 0.16

6.3.5. Cytokine Production Analysis by Spleen and Thymus Cells from Offspring of Rats fed the Different Diets in Pregnancy

Cytokine production from spleen and thymus cells was analysed from adult female tissues. Cytokine production analysis results are shown in Figure 6-4 (Spleen IFN-γ), Figure 6-5 (Thymus IFN-γ), Figure 6-6 (spleen IL-4), Figure 6-7 (Thymus IL-4), Figure 6-8 (Spleen ratio of IFN-γ to IL-4), Figure 6-9 (Thymus ratio of IFN-γ to IL-4). On the whole, there was no significant difference between levels of cytokines produced within a generation, except for two cases. At 2.5 μg / ml Con A, level of IFN-γ produced by thymus cells from the C-C group was higher than level produced by the PR-C and PR-CF groups; there was no effect at any other concentration of Con A. At 10 μg / ml Con A, ratio of IFN-γ to IL-4 produced by spleen cells was higher in the PR group than the C group in the F1 generation.

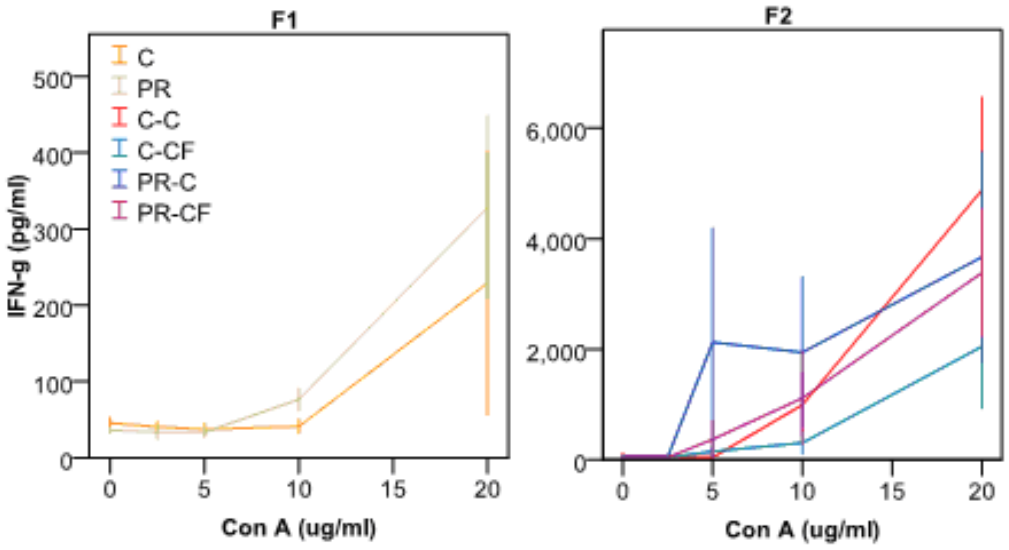

Figure 6-4: Level of IFN-γ produced by spleen cells from the F1 and F2 generations who received experimental diets in utero. There was no significant difference between cytokine level produced at any concentration of stimulant.

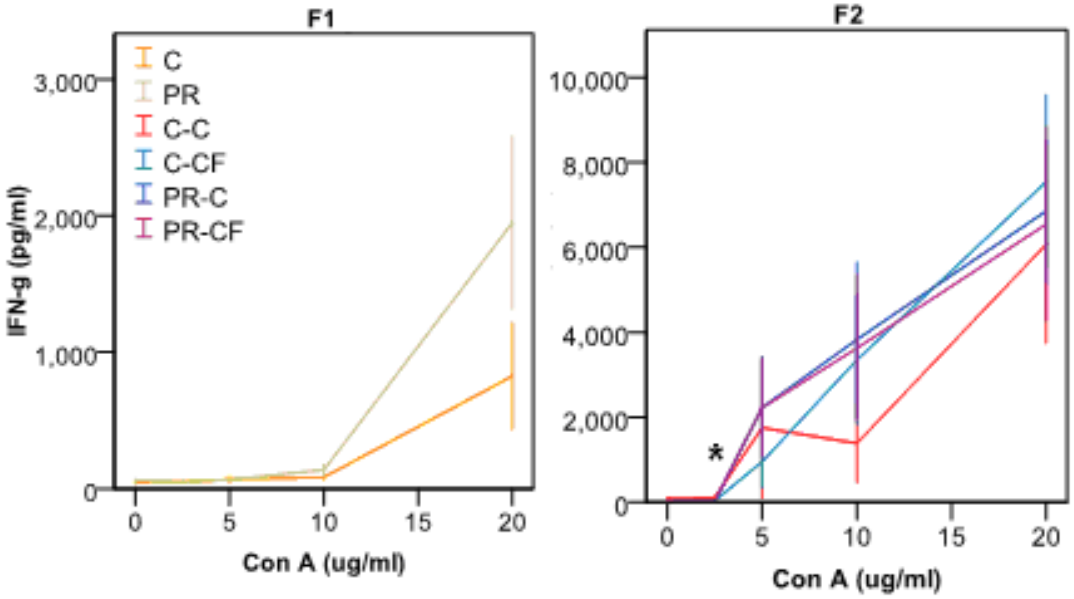

Figure 6-5: Level of IFN-γ produced by thymus cells from the F1 and F2 generations who received experimental diets in utero. At 2.5 µg / ml Con A, level of IFN-γ produced in by thymus cells from the C-C group was higher than level produced by the PR-C and PR-CF groups.

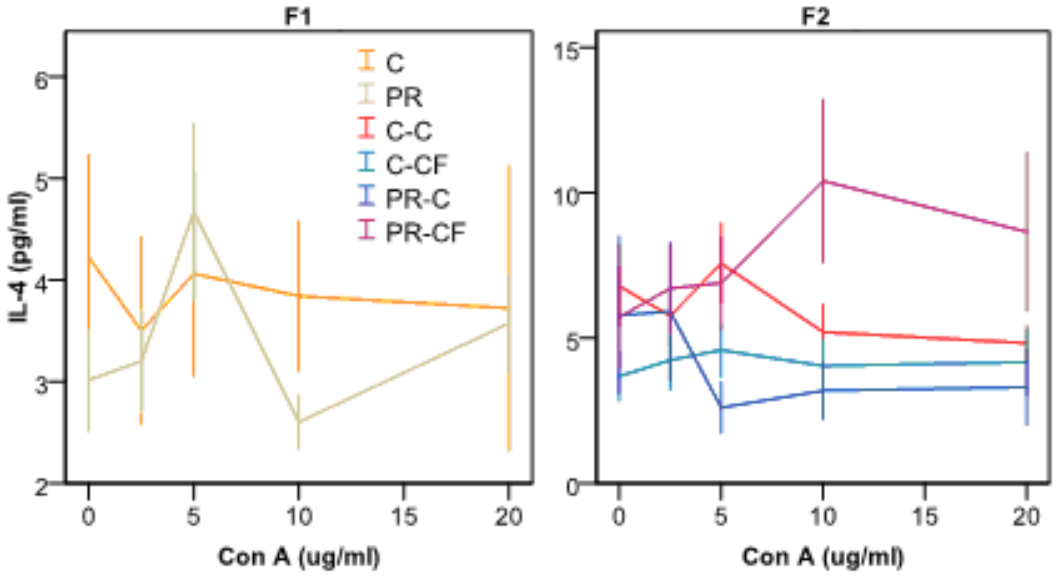

Figure 6-6: Level of IL-4 produced by spleen cells from the F1 and F2 generations who received experimental diets in utero. There was no significant difference between cytokine level produced at any concentration of stimulant.

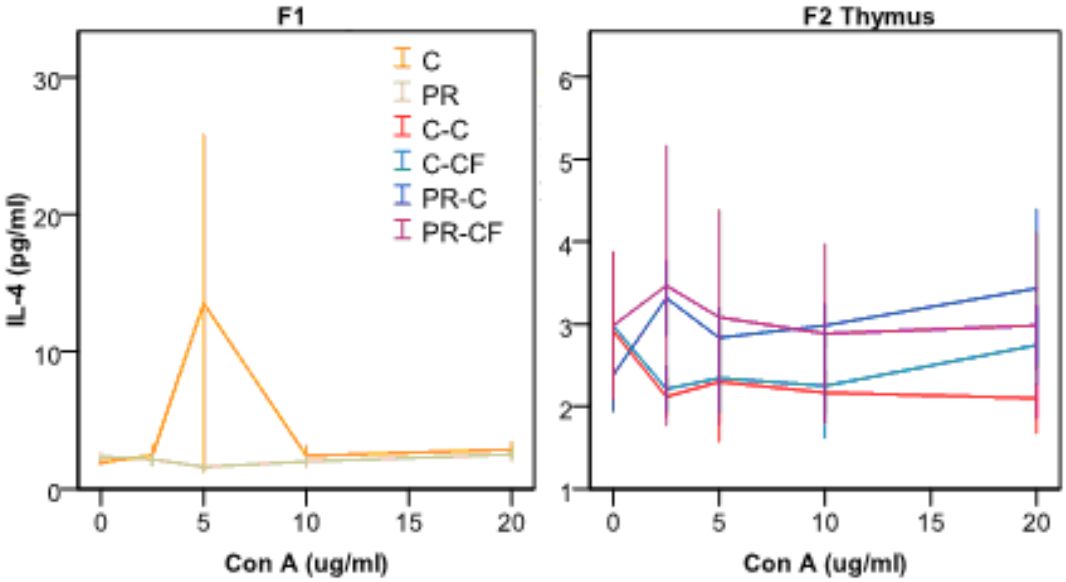

Figure 6-7: Level of IL-4 produced by thymus cells from the F1 and F2 generations who received experimental diets in utero. There was no significant difference between cytokine level produced at any concentration of stimulant.

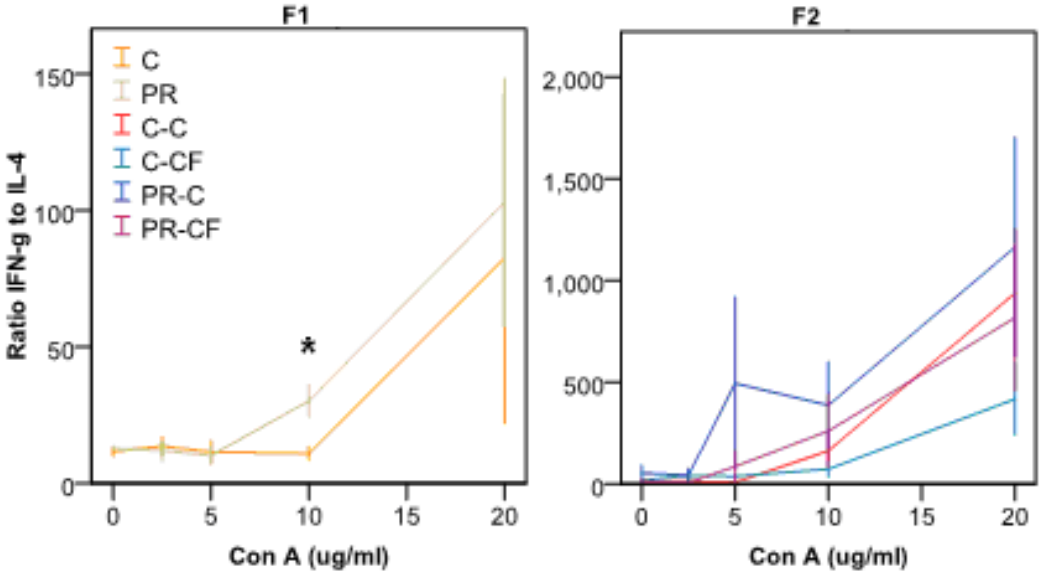

Figure 6-8: Ratio of IFN-γ to IL-4 produced by spleen cells from the F1 and F2 generations who received experimental diets in utero. At 10 µg / ml Con A, ratio of IFN-γ to IL-4 was higher in the PR group than the C group in the F1 generation.

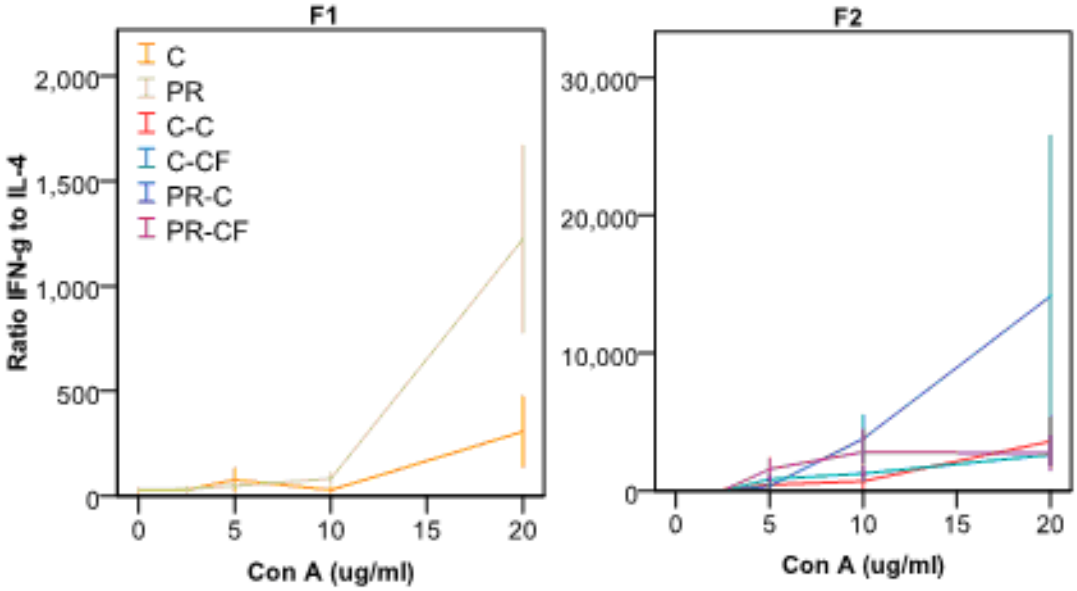

Figure 6-9: Ratio of IFN-γ to IL-4 produced by thymus cells of the F1 and F2 generation who received diets in utero. There was no significant difference between cytokine level produced at any concentration of stimulant.

6.4. Discussion

6.4.1. Summary of Findings

This study aimed to determine the effect in two generations, of diets during pregnancy of the F0 generation that provided adequate or low protein on the immune system of offspring (F1 and F2). The study set out to test the hypothesis of reduced lymphoid tissue, T_H and B-cell abundance, and altered T_H1 / T_H2 balance (determined by IL-4 / IFN-γ production) in F1 offspring as a result of protein restriction in the F0 generation, and that this effect would also be observed in the F2 generation. There was no effect of diet in pregnancy on duration of pregnancy, litter size, ratio or number of males and females in the litter. Males and F1 females showed no difference in thymus or spleen weight, but F2 females showed higher thymus weights in the C-C group compared to the C-CF and PR-C group, and higher spleen weight in the C-C group compared to the PR-CF group. There was no effect of diet on cell counts of the spleen or thymus in F1 and F2 females, or F1 males. In F2 males, however, relative (to tissue weight) and absolute cell (total) numbers were higher in the PR-C generation than the C-C and C-CF diets in spleens and thymuses. There was no effect of diet in pregnancy on cell subset abundance in females or F1 males. In the F2 male group, T_H cell abundance was higher in C-CF group than the PR-C and PR-CF groups, and B-cell abundance was higher in the PR-C group than the C-C and C-CF groups. Cytokine analysis from adult females did not show any significance, except for samples of thymocytes from the C-C group, which produced more IFN-γ when 2.5 μg / ml Con A was used as a stimulant, compared to thymocytes from the PR-C and PR-CF groups. At 10 μg / ml Con A, ratio of IFN-γ to IL-4 produced by spleen cells was higher in the PR group than the C group in the F1 generation.

6.4.2. Gestational Data

There was no effect of diet in pregnancy on duration of pregnancy, litter size, ratio or number of males and females in the litter, in either generation. This supports the findings of other studies, which have shown to be no effect of protein restriction on duration of pregnancy [267], litter size [267, 268] or ratio or number of males and females in the litter of mice [267, 268].

6.4.3. Tissue Weights of Offspring

Males and F1 females showed no difference in thymus or spleen weight, but F2 females showed greater thymus weights in the C-C group compared to the C-CF and PR-C groups, and higher spleen weight in the C-C group compared to the PR-CF group. This suggests that folate supplementation and protein restriction may be reducing tissue weight compared to a control diet, in second generation females. Therefore there may be an effect of gender or age, as the same observation was not seen in young males.

It may have been that an effect would have been observed in body weight between the PR and C groups, and the diets of the F2 generation. Body weight has been shown to be lower in new born pigs [269, 270] and rats [271, 272] as a result of protein restriction in pregnancy. In addition, there may have been a difference observed in tissue weight relative to body weight. However, body weight data was not available for analysis, and therefore lymphoid tissue weights were analysed as an alternative method of assessing the effect of protein restriction in pregnancy on physical parameters, as well as providing further insight into the effect on immune parameters.

6.4.4. Cell Counts and Subsets in the Thymus and Spleen

There was no effect of diet on cell numbers of the spleen or thymus in F1 and F2 females, or F1 males. In F2 males, however, relative (to tissue weight) and absolute (total) cell numbers in the spleens and thymuses were higher in the PR-C group than in the C-C and C-CF groups. However, this may be an artefact of analysis, due to the small sample size and large variation.

There was no effect of diet in pregnancy on cell subset abundance in females or F1 males. In the F2 male group, T_H cell abundance was higher in C-CF group than the PR-C and PR-CF groups, and B-cell abundance was higher in the PR-C group than the C-C and C-CF groups. Other methods of inducing pre-natal stress have shown an reduction in abundance of T_H cells [138, 273], proliferation of T- and B-cells [136], and reduction in serum levels of IgG [137]. The reduction in T_H cells by Gotz *et al.* [138] is in agreement with the findings of this study. B-cell abundance was shown to be increased in this study by protein restriction, whilst Kay *et al.* [136] found a reduction in B cell proliferation, and Sobrian *et al.* [137] showed a reduction in serum IgG. It may therefore be that protein restriction increases B-cell abundance, but reduces proliferation in response to a stimulant and IgG production. However, this study did not examine proliferative response or antibody production, and therefore this cannot be confirmed.

6.4.5. Cytokine Analysis

Cytokine analysis from adult females did not show any significant differences between dietary groups, except for thymocytes from the C-C group, which produced more IFN-γ when 2.5 μg / ml Con A was used as a stimulant, compared to thymocytes from the PR-C and PR-CF groups. At 10 μg / ml Con A, ratio of IFN-γ to IL-4 produced by spleen cells was higher in the PR group than the C group in the F1 generation. However, as there are only two differences in all concentrations of Con A, and these changes occur at low concentrations of stimulant, it is likely that difference may not be important, and may be due to the small sample size and large error margin.

6.4.6. Limitations

Females were required for the breeding of the subsequent generation, and as such it was not possible to analyse tissues from young female offspring. Therefore the effect of gender on any trans-generational effect on the immune system cannot be separated from effects introduced by age. It would have been preferable to have young and old males and females in order to control for both age and gender variables, but financial and operational constraints made this impossible. It is therefore impossible to determine whether any differences seen between 28 day old males and 70 day old females are due to age or gender.

6.4.7. Conclusions

It was hypothesised that protein restriction in the F0 generation would affect the immune system of the F1 and subsequently F2 generations. However, in data from this study, changes in the F1 generation were not observed, but changes to the immune system of the F2 generation were evident. For instance, protein restriction reduced T_H and B-cell abundance in the spleen and thymus of F2 males. It would be interesting to determine what the functional consequences of such a change would be on the F2 generation, and whether there had been any changes in the F1 generation that the study was not able to determine, due to the nature of the experiments carried out, such as the effect on immune cell proliferation in response to stimulant. The effect of gender on the alteration of immune system seen across subsequent generations requires further examination, however, the findings from this study add weight to the theory of programming, and suggest that physical, and possibly functional, changes to the immune system may not be observed in the immediate generation but may appear later.

Chapter 7. Summary and Concluding Remarks

7.1. Introduction

Research to date has suggested that fatty acids may affect the immune system, through their (and those of their metabolites) effects on membranes, mediators, and gene expression. However, despite the research carried out, there still exist gaps of knowledge where further research is required. In addition, programming by diet in pregnancy may affect the immune system, due to stress and/or structural and functional changes to immune cells, but whether this effect is long-lasting is uncertain. In order to address some of these gaps in knowledge of the effect of programming and fatty acids on the immune system, several experiments were carried out. These experiments aimed to examine the effects of fatty acids and / or programming of foetal physiology on different aspects and at different stages of development of the immune system, and functional consequences of these changes, in models of clinical relevance.

7.2. Summary of Experimental Data

7.2.1. Effect of Dietary Fatty Acids on the Murine Immune Response using Vaccination as an Immune Challenge (Chapter 2)

The study described in Chapter 2 investigated the effect of diets differing in fatty acid composition on the recall response in a mouse model of vaccination, using Influvac (an influenza vaccine). The study set out to test the hypothesis that altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing an increased or decreased recall immune response to Influvac. Data from measuring ear swelling, suggest that mice had an increased T_H1 response to vaccination following feeding with salmon oil, compared to linseed oil, sunflower oil or tallow, in accordance with some of the hypotheses being tested. However, analysis of plasma immunoglobulins showed no effect of diet on IgG2a levels, and plasma levels of IgG1 and spleen cell culture levels of IFN-γ, IL-4 and IL-10 were below detection limit, and therefore could not add weight to this conclusion. It may be that levels of IgG2a may have been affected by supplementation in comparison to baseline values, but this was not measured due to the size of animals at this point. Results from this chapter suggest that the *n-3* PUFAs found in salmon oil may improve the T_H1-mediated immune response and may not be detrimental to the response to immune challenges like infection as was suggested by Schwerbrock *et al.* [146]. Although the difference in DTH response between linseed oil and

salmon oil may be due to the need to convert ALA to DHA, which appears to be rate-limiting, the most important difference in fatty acid composition that is related to immune function appears to be the lower *n-6* fatty acid status that occurs with salmon oil feeding.

7.2.2. The Effect of Dietary Fatty Acids on a Mouse Model of Allergic Sensitisation (Chapter 3)

The study described in Chapter 3 aimed to determine the effect of diets differing in fatty acid composition, on the immune response in a mouse model of allergy to the egg protein ovalbumin. It set out to test the hypothesis that altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing increased or decreased susceptibility to the allergen to which the mice had been sensitised. These hypotheses were supported to some extent in that there was reduced immediate hypersensitivity in the salmon oil group and increased immediate hypersensitivity in the tallow group. However, varying the types of fatty acids in diets given to 3 to 4 week old mice for 48 days did not appear to affect airway responsiveness to ovalbumin. The increased ear swelling in animals on the tallow diet compared those receiving the soybean oil diet is reflected by an increase in IgE concentration in mice fed the tallow diet. Differences in IgE levels were not observed between other dietary groups. This may have been due to several factors, such as short feeding time (48 days) or age of the animals.

There did not appear to be changes induced in cell abundance in bronchoalveolar lavage fluid, or in cell subtypes in thymus, spleen and bone marrow, but these measurements do not determine the difference in balance of T_H1 / T_H2 cells. Examining the characteristic cytokines of these cells gave variable results. Splenocytes from mice receiving the tallow diet had increased IL-4 production in response to T-cell stimulation compared to control and sunflower oil groups, whilst there was no difference in response to ovalbumin stimulation. In addition, there was no difference observed in IFN-γ or IL-10 production in splenocytes, or IL-5 levels in BALF.

Thus, some of the diets (salmon oil, beef tallow) had effects on immediate hypersensitivity to ovalbumin in ovalbumin-sensitised mice. Beef tallow feeding also raised IgE in blood and elevated IL-4 production by anti-CD3 stimulated splenocytes. There was no effect of diet on lung function and salmon oil did not affect IgE or cytokine profiles. The diets were fed for 48 days beyond weaning and it may be that changing fatty acid availability earlier in life (i.e. pre-weaning) or a longer duration of feeding may have bigger effects. Nevertheless the results suggest that the *n-3* PUFAs found in salmon oil decrease the T_H2-mediated response to an allergen and that this is increased by a diet rich

in saturated fatty acids as found in beef tallow. The lowered T_H2 response seen with salmon oil here is consistent with the enhanced T_H1 response seen in the previous chapter.

7.2.3. Effect of Fatty Acids in Pregnancy on Markers of Immune Function in the Offspring (Chapter 4)

The study described in Chapter 4 aimed to investigate the longevity of any effect of maternal dietary fatty acids in rat pregnancy on markers of immune status, in particular the types of T- and B-lymphocytes. It set out to test the hypothesis that altering maternal fatty acid intake can cause structural changes in foetal and / or maternal immune cells, which in turn may cause long-lasting effects on immune function. This study hypothesised that changes in fatty acid intake in pregnancy would affect the fatty acid composition of maternal plasma and tissues (at the end of lactation) and of offspring plasma at weaning and beyond, and would affect the abundance of immune cells in the lymphoid tissues of the offspring. The fatty acid composition of the rat diet during pregnancy did affect the fatty acid composition of maternal plasma, liver, spleen and thymus at the end of lactation. However there was no effect on offspring fatty acid composition at weaning and beyond or on lymphoid organ immune cell subsets. Thus it was concluded that the effects of maternal fatty acids on late foetal fatty acid composition and thymus lymphocyte sub-populations [133, 217, 218] are not retained at weaning or beyond if the dams are transferred to a chow diet after giving birth. Thus, these parameters appear not to be programmed by maternal fatty acid exposures. However, *n-3* and *n-6* PUFA balance in pregnancy may affect immune markers not measured in this study, such as the balance of T_H1 and T_H2 cells, and subsequently immune reactions. In addition, the functional consequences of any changes (that were not measurable in this trial) remain to be determined. It may be of particular importance to examine these functional consequences *in vivo*, as the immune system is a complex of interacting factors and not the result of the action of one singular type of cell(s). For example, dietary intake in pregnancy may affect the development of allergy or immune response.

7.2.4. Effect of Early Exposure to Fatty Acids on Later Allergic Sensitisation Examined using a Mouse Model (Chapter 5)

The study described in Chapter 5 aimed to determine the effect of diets differing in fatty acid composition, given during gestation and / or lactation, on the immune response

in a mouse model of allergy to the egg protein ovalbumin. It was hypothesized that altering intake of fatty acids may cause structural and functional changes in the immune system of the mouse, causing them to become more or less susceptible to their allergy. In addition, mice may be more susceptible to the effects of fatty acids during gestation than lactation, if this is a critical window of fatty acid effect on immune system development. Using ear swelling in response to ovalbumin, to which the mice had been sensitised, as the outcome, animals whose dams had received an ALA-rich diet in gestation and lactation, and those that received the LA-rich diet in gestation had lower ear swelling compared to those receiving the low fat control diet for the whole period. Animals receiving the ALA-rich diet in lactation had lower ear swelling response than those receiving the LA-rich diet in gestation and lactation, or lactation alone. Despite these differences, there were no effects on lung function or immune markers measured in BALF, plasma, thymocytes, bone marrow or splenocytes. In addition, it does not appear that sensitisation was completely successful.

7.2.5. Effect of a Low Protein Diet in Pregnancy on the Immune System of Offspring in Two Generations (Chapter 6)

The study described in Chapter 6 aimed to determine the effect on the immune system over two generations of diets consumed during rat pregnancy that provided adequate or low protein. It was hypothesized that protein restriction in pregnancy would lead to reduced lymphoid tissue weight, reduce T_H and B-cell prevalence of the F1 generation, and alter the cytokine profile and that effects would also be seen in the F2 generation. However, no differences were seen between groups in the F1 generation. In contrast some effects were seen in the F2 generation. For instance, protein restriction reduced T_H and B-cell abundance in the spleen and thymus of F2 males. It would be interesting to determine what the functional consequences of such a change would be on the F2 generation, and whether there had been any changes in the F1 generation that the study was not able to determine, due to the nature of the experiments carried out, such as the effect on immune cell proliferation in response to stimulant. The effect of gender on the alteration of immune system seen across subsequent generations requires further examination, however, the findings from this study add weight to the theory of programming, and suggest that physical, and possibly functional, changes to the immune system may not be observed in the immediate generation.

7.3. Concluding Remarks

Data from Chapter 2 and Chapter 3 suggest that dietary fatty acids have the potential to alter immune responses. Salmon oil, rich in long chain *n-3* PUFAs was shown to reduce immediate hypersensitivity in T_H2-dominant animals, in comparison to linseed oil (rich in short chain *n-3* PUFAs), soybean oil (contain a balance of *n-3* and *n-6* PUFAs), sunflower oil (rich in *n-6* PUFAs), and tallow (rich in SFAs), and to increase the cell-mediated (delayed-type hypersensitivity) response in T_H1-dominant animals, in comparison to linseed oil, sunflower oil and tallow. That these responses were seen in T_H1- and T_H2-dominant animals requires further research, since response to dietary fatty acids may differ between these animals, and potentially therefore, dietary fatty acids may have different effects in humans, depending on whether they are atopic (i.e. T_H2-dominant) or not (i.e. T_H1-dominant).

Data from Chapter 4 and Chapter 5 suggest that dietary fatty acids in pregnancy may not affect the immune system of offspring later in life. However, Chapter 4 did not determine the functional consequences of any changes, and Chapter 5 was not sufficiently powered to determine functional consequences. In addition, sensitisation may not have been successful in Chapter 5. As such, further research is required to determine the effect of diet in pregnancy on immune system of offspring in later life. Data from Chapter 6 suggests, however, that programming of the immune system induced by diet in pregnancy may have effects on the immune system in the second generation that are not seen in the first. Therefore, it is possible that physiological and functional changes may have been observed if animals from Chapters 4 and 5 had been bred to a second generation. It is likely that the effects seen in Chapter 6 are due to maternal stress, as some of the effects were similar to those reported using other models of maternal stress during pregnancy.

In conclusion, whilst dietary fatty acids can alter immune responses, the longevity of effect of diet in pregnancy on the immune responses of offspring is less clear. Data detailed in this thesis suggests that there may not be an apparent effect of diet in pregnancy on immune system of the immediate generation, but there may be a longer lasting effect than has been realised to date, with effects of diet in pregnancy of the F0 generation affecting the immune system of the F2 generation. Further research is required to determine the functional consequences of any changes to the immune system induced in this model, whether dietary fatty acids could have a similar effect, and how many generations this effect of diet in pregnancy lasts for.

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Appendix One – Data from Vaccination Study

(Chapter Two)

Table 1.1: Composition of control (low fat) and experimental (high fat) diets. Taken from Special Diet Services diet composition data sheets [1, 2].

	Control	Experimental diets
Moisture (%)	10.00	10.00
Crude Oil (%)	2.71	3.36
Crude Protein (%)	14.38	18.35
Crude Fibre (%)	4.65	4.23
Ash (%)	6.00	6.27
Total Dietary Fibre (%)	17.05	15.06
Starch (%)	44.97	42.37
Sugar (%)	4.05	3.90
β-carotene (mg/kg)	0.16	1.28
Retinol (µg/kg)	2566.38	5218.35
Vitamin A (iu/kg)	8554.27	17376.38
Cholecalciferol (µg/kg)	15.54	76.94
Vitamin D (iu/kg)	621.70	3077.42
α-Tocopherol (mg/kg)	76.45	93.03
Vitamin E (iu/kg)	84.10	102.81
Vitamin B_1 (mg/kg)	8.58	15.84
Vitamin B_2 (mg/kg)	4.33	13.28
Vitamin B_6 (mg/kg)	4.81	17.65
Vitamin B_{12} (µg/kg)	7.49	78.17
Vitamin C (mg/kg)	2.59	1.80
Vitamin K (mg/kg)	10.17	185.05
Folic Acid (mg/kg)	0.79	4.30
Nicotinic Acid (mg/kg)	61.32	78.92
Pantothenic Acid (mg/kg)	20.17	25.24
Choline (mg/kg)	1080.14	899.51
Inositol (mg/kg)	2369.59	2253.88
Biotin (µg/kg)	277.13	488.74

The following are the same in both control and experimental diets							
Arginine (%)	1.19	Phenylalanine (%)	0.96	Histidine (%)	0.46	Glutamic Acid (%)	3.72
Lysine (%)	1.04	Valine (%)	0.91	Threonine (%)	0.69	Proline (%)	1.34
Methionine (%)	0.28	Tyrosine (%)	0.69	Isoleucine (%)	0.77	Serine (%)	0.78
Cystine (%)	0.29	Glycine (%)	1.55	Leucine (%)	1.46	Alanine (%)	0.21
Tryptophan (%)	0.22	Aspartic Acid (%)	1.00	Iron (mg/kg)	130.65	Fluorine (mg/kg)	9.63
Calcium (%)	0.83	Copper (mg/kg)	16.42	Chloride (%)	0.40	Cobalt (mg/kg)	494.92
Total Phosphorus (%)	0.64	Manganese (mg/kg)	91.05	Potassium (%)	0.69	Iodine (mg/kg)	390.43
Sodium (%)	0.27	Zinc (mg/kg)	86.59	Magnesium (%)	0.22	Selenium (mg/kg)	265.49

Table 1.2: Fatty acid composition of diets. Data is shown as percentage abundance. c-, cis-; dma, dimethyl acetal; t-, trans-.

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
14:0	0.09	0.17	3.34	0.18	0.21	2.17
15:0	0.00	0.03	0.28	0.02	0.03	0.39
16:0 dma	0.00	0.00	0.00	0.00	0.00	0.00
16:0	10.92	6.88	13.08	10.59	7.71	20.72
t- 16:1 n-7	0.00	0.00	0.25	0.00	0.00	0.33
16:1 n-7	0.09	0.10	4.02	0.10	0.12	2.10
17:0	0.09	0.08	0.27	0.10	0.07	1.08
Unknown 4	0.00	0.00	0.13	0.02	0.00	0.00
Unknown 5	0.04	0.04	0.18	0.04	0.04	0.05
Unknown 6	0.00	0.00	0.36	0.04	0.00	0.00
18:0	3.15	3.87	3.64	4.34	3.61	18.22
t- 18:1 n-9	0.06	0.12	0.76	0.11	0.16	0.08
18:1 n-9	22.29	18.04	20.71	20.98	29.43	30.70
18:1 n-7	1.64	0.84	2.66	1.32	1.25	1.23
19:0	0.00	0.00	0.09	0.00	0.00	0.23
c- t- 18:2 n-6	0.00	0.05	0.13	0.07	0.15	0.00
18:2 n-6	53.51	25.40	19.49	53.19	50.94	18.20
20:0	0.33	0.19	0.27	0.35	0.33	0.24
18:3 n-6	0.40	0.19	0.05	0.03	0.09	0.00
20:1 n-9	0.62	0.18	4.42	0.20	0.42	0.20
18:3 n-3	5.01	42.89	5.88	7.13	3.76	2.59
18:4 n-3	0.08	0.04	0.03	0.05	0.05	0.18
20:2 n-6	0.13	0.17	0.78	0.14	0.23	0.13
22:0	0.50	0.26	0.22	0.48	0.65	0.17
20:3 n-9	0.07	0.03	0.03	0.08	0.10	0.00
20:3 n-6	0.00	0.02	0.14	0.05	0.06	0.00
22:1 n-9	0.00	0.02	0.59	0.00	0.07	0.00
20:3 n-3	0.00	0.06	0.22	0.00	0.00	0.00
20:4 n-6	0.06	0.04	0.47	0.05	0.04	0.00
24:0	0.17	0.11	0.09	0.14	0.21	0.06
20:5 n-3	0.00	0.00	6.08	0.00	0.00	0.00
24:1 n-9	0.04	0.00	0.50	0.00	0.00	0.00
22:3 n-3	0.11	0.00	0.00	0.02	0.00	0.00
22:4 n-6	0.00	0.00	0.10	0.00	0.00	0.00
22:5 n-6	0.00	0.00	0.23	0.00	0.00	0.00
22:5 n-3	0.00	0.00	2.35	0.00	0.00	0.00
22:6 n-3	0.00	0.00	7.51	0.00	0.00	0.00
n-3	5.20	42.98	22.08	7.21	3.81	2.77
n-6	54.66	25.89	21.81	53.58	51.68	18.65
n-3 : n-6	9.51	166.00	101.22	13.45	7.37	14.84

Table 1.3: Fatty acid composition of erythrocytes of mice fed the different diets for 37 ± 1 days. Data is shown as percentage abundance. c-, cis-; dma, dimethyl acetal; t-, trans-.

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
14:0	0.21 ± 0.01^{b}	0.23 ± 0.02^{b}	0.44 ± 0.02^{c}	0.13 ± 0.02^{a}	0.13 ± 0.02^{a}	0.23 ± 0.02^{b}
15:0	0.11 ± 0.01^{a}	0.14 ± 0.02^{a}	0.29 ± 0.01^{c}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.21 ± 0.01^{b}
16:0 dma	1.34 ± 0.11	1.13 ± 0.08	1.18 ± 0.08	1.15 ± 0.07	1.01 ± 0.06	1.06 ± 0.10
16:0	38.21 ± 0.79^{bc}	33.24 ± 2.57^{ab}	40.03 ± 1.07^{c}	34.59 ± 1.26^{abc}	35.11 ± 1.37^{abc}	31.40 ± 1.18^{a}
t- 16:1 n-7	0.09 ± 0.01^{a}	0.05 ± 0.02^{a}	0.10 ± 0.00^{a}	0.05 ± 0.02^{a}	0.09 ± 0.01^{a}	0.28 ± 0.02^{b}
16:1 n-7	0.71 ± 0.04^{b}	0.31 ± 0.04^{a}	0.55 ± 0.02^{b}	0.20 ± 0.00^{a}	0.26 ± 0.02^{a}	0.65 ± 0.09^{b}
17:0	0.31 ± 0.01^{a}	0.41 ± 0.04^{b}	0.56 ± 0.02^{c}	0.41 ± 0.01^{b}	0.36 ± 0.02^{ab}	0.78 ± 0.02^{d}
Unknown 4	0.76 ± 0.06	0.80 ± 0.05	0.74 ± 0.05	0.89 ± 0.04	0.78 ± 0.05	0.90 ± 0.07
Unknown 5	0.41 ± 0.02^{c}	0.31 ± 0.06^{bc}	0.23 ± 0.04^{ab}	0.34 ± 0.02^{bc}	0.33 ± 0.06^{bc}	0.13 ± 0.04^{a}
Unknown 6	0.48 ± 0.13^{c}	0.08 ± 0.03^{a}	0.13 ± 0.03^{ab}	0.36 ± 0.04^{bc}	0.59 ± 0.08^{c}	0.09 ± 0.02^{ab}
18:0	11.08 ± 0.34^{a}	14.46 ± 0.96^{b}	11.50 ± 0.43^{a}	14.05 ± 0.40^{b}	13.29 ± 0.50^{ab}	13.23 ± 0.32^{ab}
t- 18:1 n-9	0.09 ± 0.02^{a}	0.08 ± 0.02^{a}	0.30 ± 0.00^{b}	0.10 ± 0.02^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}
18:1 n-9	12.44 ± 0.22^{b}	13.01 ± 0.68^{b}	12.20 ± 0.11^{b}	10.33 ± 0.20^{a}	12.33 ± 0.20^{b}	15.44 ± 0.20^{c}
18:1 n-7	2.15 ± 0.04^{d}	1.18 ± 0.08^{a}	1.94 ± 0.03^{c}	1.20 ± 0.03^{a}	1.24 ± 0.03^{a}	1.55 ± 0.06^{b}
19:0	0.15 ± 0.02^{a}	0.14 ± 0.02^{a}	0.20 ± 0.00^{ab}	0.20 ± 0.00^{ab}	0.23 ± 0.02^{b}	0.15 ± 0.02^{a}
t- t- 18:2 n-6	0.26 ± 0.10^{b}	0.15 ± 0.04^{ab}	0.00 ± 0.00^{a}	0.03 ± 0.02^{a}	0.00 ± 0.00^{a}	0.04 ± 0.03^{a}
c- t- 18:2 n-6	0.16 ± 0.07	0.06 ± 0.05	0.08 ± 0.02	0.21 ± 0.04	0.18 ± 0.04	0.14 ± 0.03
t- c- 18:2 n-6	0.03 ± 0.02^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.03 ± 0.02^{ab}	0.06 ± 0.02^{b}
18:2 n-6	10.49 ± 0.34^{ab}	14.69 ± 0.83^{c}	8.76 ± 0.51^{a}	15.26 ± 0.75^{c}	12.64 ± 0.80^{bc}	11.43 ± 0.38^{ab}
20:0	0.39 ± 0.01^{a}	0.45 ± 0.03^{ab}	0.38 ± 0.02^{a}	0.48 ± 0.02^{b}	0.45 ± 0.03^{ab}	0.43 ± 0.02^{ab}
18:3 n-6	0.08 ± 0.02^{abc}	0.03 ± 0.02^{ab}	0.00 ± 0.00^{a}	0.10 ± 0.03^{bc}	0.11 ± 0.02^{c}	0.08 ± 0.02^{abc}
20:1 n-9	0.43 ± 0.02^{b}	0.34 ± 0.02^{a}	1.03 ± 0.03^{c}	0.36 ± 0.02^{ab}	0.41 ± 0.01^{b}	0.41 ± 0.01^{b}
18:3 n-3	0.23 ± 0.02^{a}	1.59 ± 0.10^{b}	0.21 ± 0.01^{a}	0.28 ± 0.03^{a}	0.16 ± 0.02^{a}	0.20 ± 0.00^{a}
Unknown 7	0.00 ± 0.00	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.02
18:4 n-3	0.23 ± 0.03	0.21 ± 0.06	0.20 ± 0.05	0.15 ± 0.03	0.20 ± 0.05	0.26 ± 0.03
20:2 n-6	0.45 ± 0.03^{bc}	0.40 ± 0.03^{abc}	0.31 ± 0.02^{a}	0.51 ± 0.03^{c}	0.49 ± 0.04^{c}	0.35 ± 0.02^{ab}
22:0	1.51 ± 0.09	1.46 ± 0.16	1.41 ± 0.13	1.63 ± 0.09	1.59 ± 0.15	1.91 ± 0.15
20:3 n-9	0.03 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	0.05 ± 0.02
20:3 n-6	1.01 ± 0.06^{c}	0.65 ± 0.08^{b}	0.29 ± 0.03^{a}	0.93 ± 0.07^{bc}	0.96 ± 0.11^{bc}	1.21 ± 0.10^{c}
22:1 n-9	0.11 ± 0.01^{a}	0.13 ± 0.02^{a}	0.23 ± 0.02^{b}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.11 ± 0.01^{a}
20:3 n-3	0.10 ± 0.00^{a}	0.19 ± 0.02^{b}	0.09 ± 0.01^{a}	0.05 ± 0.02^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}
20:4 n-6	6.68 ± 0.55^{b}	2.83 ± 0.31^{a}	2.13 ± 0.20^{a}	6.24 ± 0.61^{b}	7.03 ± 0.89^{b}	7.56 ± 0.92^{b}
22:2 n-x	0.04 ± 0.02^{a}	0.03 ± 0.02^{a}	0.00 ± 0.00^{a}	0.10 ± 0.00^{b}	0.09 ± 0.01^{b}	0.00 ± 0.00^{a}

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
24:0	2.59 ± 0.13^{ab}	3.20 ± 0.32^{b}	2.28 ± 0.10^{a}	3.06 ± 0.18^{ab}	3.04 ± 0.18^{ab}	2.55 ± 0.13^{ab}
Unknown 9	0.28 ± 0.05	0.14 ± 0.05	0.18 ± 0.06	0.23 ± 0.03	0.25 ± 0.04	0.10 ± 0.04
20:5 n-3	0.11 ± 0.03^{a}	1.76 ± 0.22^{b}	2.40 ± 0.25^{c}	0.13 ± 0.04^{a}	0.01 ± 0.01^{a}	0.19 ± 0.04^{a}
24:1 n-9	1.71 ± 0.08^{ab}	1.39 ± 0.12^{a}	3.84 ± 0.10^{c}	1.45 ± 0.08^{a}	1.86 ± 0.09^{b}	1.64 ± 0.07^{ab}
22:3 n-3	0.09 ± 0.01	0.06 ± 0.02	0.09 ± 0.01	0.08 ± 0.02	0.09 ± 0.01	0.08 ± 0.02
22:4 n-6	0.96 ± 0.09^{b}	0.18 ± 0.03^{a}	0.13 ± 0.03^{a}	0.76 ± 0.07^{b}	1.03 ± 0.14^{b}	0.94 ± 0.15^{b}
22:5 n-6	0.81 ± 0.07^{b}	0.43 ± 0.04^{a}	0.38 ± 0.05^{a}	0.90 ± 0.04^{b}	0.93 ± 0.15^{b}	0.61 ± 0.07^{ab}
22:5 n-3	0.39 ± 0.06^{a}	1.39 ± 0.22^{b}	1.03 ± 0.13^{b}	0.48 ± 0.07^{a}	0.28 ± 0.04^{a}	0.45 ± 0.08^{a}
22:6 n-3	2.14 ± 0.20^{a}	2.56 ± 0.36^{a}	4.15 ± 0.52^{b}	2.38 ± 0.30^{a}	1.91 ± 0.25^{a}	2.78 ± 0.36^{ab}
n-3	3.32 ± 0.27^{ab}	7.79 ± 0.85^{c}	8.15 ± 0.87^{c}	3.54 ± 0.40^{ab}	2.72 ± 0.25^{a}	4.04 ± 0.45^{b}
n-6	21.00 ± 1.08^{b}	19.41 ± 1.25^{b}	12.10 ± 0.79^{a}	24.97 ± 1.57^{b}	23.44 ± 1.96^{b}	22.41 ± 1.36^{b}
n-3 : n-6	0.15 ± 0.02^{ab}	0.37 ± 0.03^{c}	0.65 ± 0.04^{d}	0.12 ± 0.02^{a}	0.10 ± 0.00^{a}	0.19 ± 0.01^{b}

References

1. Special Diet Services, *Rat and Mouse No. 1 Maintenance (Catalogue revision 4)*. 2004a.
2. Special Diet Services, *Rat and Mouse Breeder and Grower (Catalogue revision 4a)*. 2004b.

Appendix Two – Data from Allergic Sensitisation Study (Chapter 3).

Table 2-1: Fatty acid composition of erythrocytes of mice fed the experimental diets for 48 ± 1 days. Data is shown as percentage abundance. c-, cis-; dma, dimethyl acetal; t-, trans-.

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
14:0	0.18 ± 0.02^{ab}	0.17 ± 0.02^{ab}	0.25 ± 0.06^{b}	0.10 ± 0.00^{a}	0.12 ± 0.02^{a}	0.28 ± 0.03^{b}
15:0	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.18 ± 0.02^{b}	0.10 ± 0.00^{a}	0.12 ± 0.02^{a}	0.20 ± 0.00^{b}
16:0 dma	1.97 ± 0.03^{b}	1.73 ± 0.03^{ab}	1.67 ± 0.08^{a}	1.75 ± 0.02^{ab}	1.58 ± 0.02^{a}	1.80 ± 0.08^{ab}
16:0	30.33 ± 0.24	30.08 ± 0.36	29.25 ± 0.88	29.20 ± 0.28	27.78 ± 0.47	28.95 ± 0.97
t- 16:1 n-7	0.10 ± 0.00^{abc}	0.00 ± 0.00^{a}	0.12 ± 0.03^{bc}	0.00 ± 0.00^{a}	0.04 ± 0.04^{ab}	0.18 ± 0.03^{c}
16:1 n-7	0.55 ± 0.04^{b}	0.27 ± 0.02^{a}	0.55 ± 0.08^{b}	0.18 ± 0.02^{a}	0.26 ± 0.06^{a}	0.63 ± 0.03^{b}
17:0	0.27 ± 0.02	0.33 ± 0.02	0.52 ± 0.07	0.30 ± 0.00	0.38 ± 0.08	0.48 ± 0.08
Unknown 4	1.07 ± 0.02^{a}	1.20 ± 0.00^{ab}	1.23 ± 0.10^{ab}	1.28 ± 0.02^{b}	1.20 ± 0.05^{ab}	1.26 ± 0.03^{ab}
Unknown 5	0.70 ± 0.00^{b}	0.65 ± 0.02^{b}	0.18 ± 0.08^{a}	0.57 ± 0.02^{b}	0.62 ± 0.13^{b}	0.09 ± 0.01^{a}
Unknown 6	0.15 ± 0.02	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.14 ± 0.02
18:0	9.32 ± 0.14^{a}	10.87 ± 0.08^{bc}	10.67 ± 0.44^{bc}	11.25 ± 0.17^{c}	10.72 ± 0.08^{b}	10.08 ± 0.27^{ab}
t- 18:1 n-9	0.10 ± 0.00^{a}	0.13 ± 0.02^{a}	0.40 ± 0.07^{bc}	0.20 ± 0.00^{ab}	0.26 ± 0.09^{ab}	0.55 ± 0.06^{c}
18:1 n-9	11.92 ± 0.19^{ab}	12.75 ± 0.06^{bc}	13.60 ± 0.41^{bc}	10.08 ± 0.09^{a}	12.50 ± 0.66^{b}	14.40 ± 0.61^{c}
18:1 n-7	1.63 ± 0.10^{b}	0.85 ± 0.02^{a}	1.32 ± 0.12^{b}	0.92 ± 0.02^{a}	0.96 ± 0.02^{a}	1.38 ± 0.08^{b}
19:0	0.10 ± 0.00	0.13 ± 0.02	0.17 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.15 ± 0.02
c- t- 18:2 n-6	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.08 ± 0.02	0.10 ± 0.00	0.16 ± 0.07
18:2 n-6	10.65 ± 0.21^{a}	13.57 ± 0.29^{b}	9.80 ± 0.59^{a}	14.37 ± 0.30^{b}	13.30 ± 0.35^{b}	10.13 ± 0.58^{a}
20:0	0.37 ± 0.02	0.40 ± 0.00	0.40 ± 0.04	0.42 ± 0.02	0.38 ± 0.02	0.36 ± 0.03
18:3 n-6	0.03 ± 0.02	0.00 ± 0.00	0.02 ± 0.02	0.05 ± 0.02	0.02 ± 0.02	0.03 ± 0.02
20:1 n-9	0.45 ± 0.02^{ab}	0.33 ± 0.02^{a}	0.60 ± 0.08^{b}	0.33 ± 0.02^{a}	0.40 ± 0.00^{ab}	0.48 ± 0.08^{ab}
18:3 n-3	0.22 ± 0.02^{a}	1.62 ± 0.07^{b}	0.27 ± 0.09^{a}	0.28 ± 0.02^{a}	0.20 ± 0.00^{a}	0.24 ± 0.02^{a}
18:4 n-3	0.07 ± 0.02^{ab}	0.00 ± 0.00^{a}	0.08 ± 0.02^{b}	0.00 ± 0.00^{a}	0.02 ± 0.02^{ab}	0.05 ± 0.02^{ab}
20:2 n-6	0.40 ± 0.00^{ab}	0.40 ± 0.00^{ab}	0.35 ± 0.03^{a}	0.52 ± 0.02^{c}	0.48 ± 0.05^{bc}	0.34 ± 0.03^{a}
22:0	1.35 ± 0.05	1.25 ± 0.05	1.42 ± 0.17	1.30 ± 0.05	1.44 ± 0.08	1.38 ± 0.18
20:3 n-9	0.10 ± 0.00	0.08 ± 0.02	0.07 ± 0.02	0.10 ± 0.00	0.10 ± 0.00	0.05 ± 0.02
20:3 n-6	1.12 ± 0.04	0.90 ± 0.03	0.90 ± 0.13	1.17 ± 0.03	1.08 ± 0.02	0.88 ± 0.13
22:1 n-9	0.10 ± 0.00	0.10 ± 0.00	0.12 ± 0.02	0.10 ± 0.00	0.12 ± 0.02	0.14 ± 0.02
20:3 n-3	0.10 ± 0.00^{a}	0.30 ± 0.00^{b}	0.10 ± 0.00^{a}	0.07 ± 0.02^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}
20:4 n-6	12.70 ± 0.18^{c}	5.38 ± 0.12^{a}	8.98 ± 1.65^{abc}	11.78 ± 0.23^{bc}	12.42 ± 0.41^{c}	7.83 ± 1.42^{ab}
24:0	1.95 ± 0.10^{ab}	2.52 ± 0.09^{c}	1.75 ± 0.15^{ab}	2.17 ± 0.10^{bc}	2.14 ± 0.10^{abc}	1.70 ± 0.07^{a}
20:5 n-3	0.20 ± 0.00^{a}	2.20 ± 0.07^{b}	1.72 ± 0.71^{ab}	0.20 ± 0.00^{a}	0.12 ± 0.02^{a}	1.93 ± 0.63^{ab}
24:1 n-9	1.77 ± 0.07^{abc}	1.40 ± 0.06^{ab}	2.10 ± 0.22^{bc}	1.25 ± 0.06^{a}	1.68 ± 0.07^{abc}	2.35 ± 0.24^{c}

	Control	Linseed oil	Salmon oil	Soybean oil	Sunflower oil	Tallow
22:3 n-3	0.10 ± 0.00^{b}	0.10 ± 0.00^{b}	0.10 ± 0.00^{b}	0.03 ± 0.02^{a}	0.06 ± 0.02^{ab}	0.10 ± 0.00^{b}
22:4 n-6	1.88 ± 0.04^{c}	0.32 ± 0.02^{a}	1.20 ± 0.30^{bc}	1.65 ± 0.02^{bc}	1.94 ± 0.09^{c}	0.88 ± 0.26^{ab}
22:5 n-6	2.13 ± 0.05^{c}	0.55 ± 0.02^{a}	1.35 ± 0.27^{b}	1.52 ± 0.03^{bc}	2.02 ± 0.23^{c}	0.89 ± 0.12^{ab}
22:5 n-3	0.65 ± 0.02^{ab}	2.65 ± 0.05^{c}	1.25 ± 0.33^{ab}	0.78 ± 0.02^{ab}	0.52 ± 0.05^{a}	1.63 ± 0.34^{b}
22:6 n-3	4.93 ± 0.07	6.47 ± 0.35	6.82 ± 1.07	5.55 ± 0.07	4.48 ± 0.11	7.56 ± 1.03
n-3	6.25 ± 0.10^{ab}	13.35 ± 0.32^{c}	10.32 ± 2.15^{abc}	6.92 ± 0.07^{ab}	5.42 ± 0.20^{a}	11.54 ± 1.99^{bc}
n-6	29.05 ± 0.16^{bc}	21.25 ± 0.22^{a}	22.78 ± 2.34^{ab}	31.15 ± 0.16^{c}	31.40 ± 0.77^{c}	21.20 ± 2.32^{a}
n-3 : n-6	0.20 ± 0.00^{a}	0.63 ± 0.02^{b}	0.55 ± 0.18^{b}	0.20 ± 0.00^{a}	0.20 ± 0.00^{a}	0.66 ± 0.18^{b}

Appendix Three – Data from Diet in Pregnancy Study

(Chapter 4)

Table 3-1: Fatty acid profile in CE fraction of maternal plasma, taken at weaning (3 weeks after birth of offspring). Results are shown as mean ± SEM. Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
12:0	0.15 ± 0.09	0.25 ± 0.25	0.21 ± 0.16
14:0	0.24 ± 0.06	0.17 ± 0.07	0.24 ± 0.06
16:0	9.07 ± 0.55	8.22 ± 0.71	9.04 ± 1.20
16:1 *n-7*	4.49 ± 0.64	3.49 ± 0.46	3.15 ± 0.41
18:0	2.22 ± 0.36	2.11 ± 0.42	6.60 ± 4.42
18:1 *n-9*	16.22 ± 1.55	13.26 ± 1.68	12.71 ± 2.23
trans 18:1 *n-9*	1.00 ± 0.07	1.17 ± 0.13	0.89 ± 0.12
18:2 *n-6*	22.99 ± 0.75[b]	21.79 ± 0.81[ab]	18.28 ± 1.58[a]
18:3 *n-6*	3.37 ± 0.32	2.18 ± 0.27	2.53 ± 0.44
18:3 *n-3*	0.19 ± 0.08	0.36 ± 0.03	0.25 ± 0.09
20:0	0.00 ± 0.00	0.00 ± 0.00	0.36 ± 0.24
20:1 *n-9*	0.38 ± 0.38	0.22 ± 0.14	0.17 ± 0.13
20:2 *n-6*	1.55 ± 0.25	1.07 ± 0.34	0.92 ± 0.37
20:3 *n-6*	0.96 ± 0.15	0.52 ± 0.22	0.85 ± 0.15
20:4 *n-6*	28.60 ± 2.36	39.43 ± 3.04	39.39 ± 5.23
20:3 *n-9*	0.00 ± 0.00	7.60 ± 7.60	10.49 ± 10.49
22:1 *n-9*	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.15
20:5 *n-3*	5.47 ± 0.29[b]	2.62 ± 0.49[a]	2.22 ± 0.39[a]
22:2 *n-9*	1.39 ± 0.41	1.95 ± 0.59	1.21 ± 0.40
22:5 *n-3*	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00
22:6 *n-3*	1.71 ± 0.40	1.09 ± 0.28	0.84 ± 0.26
n-3	7.38 ± 0.55[b]	4.17 ± 0.85[a]	3.30 ± 0.54[a]
n-6	28.87 ± 0.87	33.16 ± 7.78	33.07 ± 9.59
Ratio *n-3*:*n-6*	0.26 ± 0.02[b]	0.14 ± 0.03[a]	0.12 ± 0.03[a]

Table 3.2: Fatty acid profile in NEFA fraction of maternal plasma, taken at weaning (3 weeks after birth of offspring). There was no difference in fatty acid profile between different dietary groups. For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
12:0	1.44 ± 0.84	0.53 ± 0.38	0.79 ± 0.49
14:0	1.79 ± 0.19	1.83 ± 0.19	2.67 ± 0.71
16:0	31.41 ± 1.02	30.14 ± 3.75	33.00 ± 1.39
16:1 *n-7*	2.80 ± 0.58	2.06 ± 0.77	1.99 ± 0.77
18:0	24.12 ± 4.29	24.13 ± 4.85	33.52 ± 3.70
18:1 *n-9*	15.32 ± 3.61	10.06 ± 2.35	8.66 ± 1.97
trans 18:1 *n-9*	1.50 ± 0.24	2.47 ± 0.74	0.77 ± 0.77
18:2 *n-6*	8.66 ± 1.15	15.91 ± 9.24	6.55 ± 0.78
18:3 *n-6*	1.22 ± 0.37	1.58 ± 0.46	1.62 ± 0.38
18:3 *n-3*	1.16 ± 0.30	1.73 ± 0.32	1.07 ± 0.38
20:0	0.21 ± 0.14	0.00 ± 0.00	0.19 ± 0.19
20:1 *n-9*	1.06 ± 0.40	0.98 ± 0.42	0.70 ± 0.44
20:2 *n-6*	0.67 ± 0.67	0.00 ± 0.00	0.00 ± 0.00
20:3 *n-6*	0.66 ± 0.27	0.98 ± 0.54	1.26 ± 0.65
20:4 *n-6*	3.05 ± 0.70	1.81 ± 0.72	3.88 ± 0.82
20:3 *n-9*	0.00 ± 0.00	0.40 ± 0.40	0.00 ± 0.00
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 *n-9*	0.11 ± 0.11	0.78 ± 0.48	0.09 ± 0.09
20:5 *n-3*	1.18 ± 0.66	0.90 ± 0.42	0.23 ± 0.23
22:2 *n-9*	0.82 ± 0.82	0.42 ± 0.31	0.00 ± 0.00
22:4 *n-6*	0.67 ± 0.17	1.07 ± 0.55	0.74 ± 0.42
24:0	0.83 ± 0.29	0.78 ± 0.35	0.43 ± 0.27
24:1 *n-9*	0.16 ± 0.16	0.56 ± 0.28	0.29 ± 0.19
22:5 *n-3*	0.95 ± 0.52	0.22 ± 0.15	1.24 ± 0.74
22:6 *n-3*	0.21 ± 0.16	0.65 ± 0.42	0.30 ± 0.30
n-3	3.51 ± 0.88	3.50 ± 0.48	2.84 ± 0.95
n-6	15.76 ± 0.97	22.15 ± 8.49	14.48 ± 1.21
Ratio *n-3*:*n-6*	0.22 ± 0.05	0.22 ± 0.05	0.22 ± 0.09

Table 3.3: Fatty acid profile in PC fraction of maternal plasma, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
14:0	0.26 ± 0.03	0.31 ± 0.07	0.25 ± 0.01
16:0	23.76 ± 0.39	23.20 ± 0.97	23.41 ± 0.47
16:1 *n-7*	1.08 ± 0.10	1.20 ± 0.24	1.01 ± 0.10
18:0	22.36 ± 0.31	23.09 ± 1.22	23.68 ± 1.09
18:1 *n-9*	10.92 ± 0.69	9.00 ± 0.99	10.11 ± 0.69
trans 18:1 *n-9*	1.52 ± 0.05[a]	2.05 ± 0.08[b]	1.57 ± 0.08[a]
18:2 *n-6*	19.90 ± 0.47	20.51 ± 0.65	19.75 ± 0.87
18:3 *n-6*	0.42 ± 0.03[b]	0.21 ± 0.06[a]	0.40 ± 0.03[b]
18:3 *n-3*	0.07 ± 0.03	0.02 ± 0.02	0.14 ± 0.06
20:0	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00
20:1 *n-9*	0.05 ± 0.04	0.03 ± 0.03	0.09 ± 0.06
20:2 *n-6*	1.10 ± 0.21	0.75 ± 0.20	0.80 ± 0.18
20:3 *n-6*	2.23 ± 0.10[b]	1.63 ± 0.17[a]	2.01 ± 0.15[b]
20:4 *n-6*	10.48 ± 0.91	13.87 ± 1.36	12.54 ± 1.22
20:5 *n-3*	1.34 ± 0.12[b]	0.55 ± 0.17[a]	0.85 ± 0.18[ab]
22:4 *n-6*	0.11 ± 0.07	0.05 ± 0.05	0.10 ± 0.06
24:0	0.30 ± 0.16	0.61 ± 0.13	0.80 ± 0.20
24:1 *n-9*	0.50 ± 0.25	0.23 ± 0.23	0.00 ± 0.00
22:5 *n-3*	2.13 ± 0.82	1.03 ± 0.30	1.14 ± 0.28
22:6 *n-3*	1.48 ± 0.61	1.65 ± 0.59	1.33 ± 0.89
n-3	5.01 ± 0.65	3.24 ± 0.62	3.47 ± 0.97
n-6	34.52 ± 1.10	37.63 ± 1.34	36.41 ± 1.67
Ratio *n-3*:*n-6*	0.15 ± 0.02	0.09 ± 0.02	0.10 ± 0.03

Table 3.4: Fatty acid profile in TAG fraction of maternal plasma, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
14:0	1.02 ± 0.11	1.04 ± 0.14	0.82 ± 0.09
16:0	29.03 ± 1.66	25.71 ± 0.72	28.77 ± 1.21
16:1 *n-7*	4.78 ± 0.29	4.87 ± 0.48	4.43 ± 0.23
18:0	4.15 ± 0.61	3.70 ± 0.34	3.88 ± 0.38
18:1 *n-9*	38.48 ± 1.30	33.75 ± 1.17	36.83 ± 2.46
trans 18:1 *n-9*	3.25 ± 0.19^{ab}	4.07 ± 0.23^{b}	3.10 ± 0.32^{a}
18:2 *n-6*	15.00 ± 1.50	19.84 ± 1.53	15.87 ± 1.64
18:3 *n-6*	0.54 ± 0.06	0.63 ± 0.10	0.43 ± 0.09
18:3 *n-3*	1.21 ± 0.17	1.47 ± 0.19	1.18 ± 0.21
20:0	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.02
20:1 *n-9*	0.18 ± 0.06	0.61 ± 0.21	0.20 ± 0.08
20:2 *n-6*	0.34 ± 0.07	0.46 ± 0.20	0.37 ± 0.09
20:3 *n-6*	0.30 ± 0.11	0.58 ± 0.28	0.32 ± 0.13
20:4 *n-6*	0.95 ± 0.09	2.07 ± 0.27	2.41 ± 0.81
20:5 *n-3*	0.24 ± 0.11	0.52 ± 0.22	0.28 ± 0.09
22:4 *n-6*	0.00 ± 0.00	0.06 ± 0.06	0.20 ± 0.20
24:0	0.00 ± 0.00	0.04 ± 0.03	0.14 ± 0.14
24:1 *n-9*	0.08 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
22:5 *n-3*	0.31 ± 0.15	0.24 ± 0.19	0.54 ± 0.54
22:6 *n-3*	0.17 ± 0.08	0.35 ± 0.16	0.22 ± 0.17
n-3	1.92 ± 0.26	2.57 ± 0.36	2.21 ± 0.65
n-6	17.12 ± 1.64	23.68 ± 1.48	19.74 ± 2.20
Ratio *n-3*: *n-6*	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.02

Table 3.5: Fatty acid profile in CE fraction of maternal liver, taken at weaning (3 weeks after birth of offspring). There were no statistical differences between any dietary groups. For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	4.82 ± 0.24	5.22 ± 0.30	4.56 ± 0.36
12:0	1.58 ± 0.19	1.60 ± 0.18	1.57 ± 0.20
14:0	1.08 ± 0.11	1.46 ± 0.24	0.86 ± 0.05
16:0	6.31 ± 1.39	9.79 ± 2.18	7.58 ± 2.47
16:1 n-7	0.89 ± 0.16	1.10 ± 0.26	0.77 ± 0.10
18:0	5.28 ± 0.13	4.83 ± 0.25	5.24 ± 0.20
18:1 n-9	9.12 ± 0.13	8.72 ± 0.62	8.97 ± 0.44
trans 18:1 n-9	0.62 ± 0.18	2.04 ± 1.50	0.44 ± 0.09
18:2 n-6	7.64 ± 0.55	7.06 ± 0.83	7.67 ± 0.36
18:3 n-6	12.03 ± 0.28	11.01 ± 0.49	11.73 ± 0.41
18:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:0	0.58 ± 0.12	0.39 ± 0.16	0.37 ± 0.12
20:1 n-9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:2 n-6	0.77 ± 0.13	0.74 ± 0.10	0.79 ± 0.12
20:3 n-6	0.97 ± 0.24	0.56 ± 0.37	1.35 ± 0.27
20:4 n-6	16.28 ± 0.39	15.24 ± 0.62	16.05 ± 0.66
22:0	1.08 ± 0.23	0.88 ± 0.50	1.83 ± 0.47
22:1 n-9 / 20:4 n-3	0.00 ± 0.00	2.83 ± 2.83	0.00 ± 0.00
20:5 n-3	15.53 ± 0.37	11.61 ± 2.69	14.68 ± 0.52
24:0	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00
22:4 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:5 n-6	4.12 ± 0.20	3.85 ± 0.50	4.93 ± 0.38
24:1 n-9	0.48 ± 0.48	0.00 ± 0.00	0.00 ± 0.00
22:5 n-3	3.40 ± 0.54	2.76 ± 0.26	2.82 ± 0.40
22:6 n-3	7.41 ± 0.49	7.99 ± 0.52	7.78 ± 0.47
n-3	26.35 ± 1.15	25.19 ± 0.88	25.28 ± 1.06
n-6	41.82 ± 0.47	38.46 ± 2.04	42.52 ± 1.47
n-3 : n-6	0.63 ± 0.03	0.66 ± 0.02	0.60 ± 0.02

Table 3.6: Fatty acid profile in NEFA fraction of maternal liver, taken at weaning (3 weeks after birth of offspring). There were no statistical differences between any dietary groups. For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	1.17 ± 0.15	1.29 ± 0.12	1.25 ± 0.14
16:0	26.02 ± 2.36	22.45 ± 1.94	25.39 ± 0.98
16:1 n-7	4.67 ± 0.69	4.40 ± 0.72	4.43 ± 0.28
18:0	11.97 ± 1.83	13.63 ± 0.33	14.07 ± 1.22
18:1 n-9	23.32 ± 3.83	17.68 ± 2.38	19.14 ± 2.27
trans 18:1 n-9	4.48 ± 0.90	4.42 ± 0.26	3.85 ± 0.50
18:2 n-6	7.01 ± 1.66	6.51 ± 2.15	6.30 ± 2.05
18:3 n-6	1.31 ± 1.31	3.98 ± 2.53	2.13 ± 1.35
18:3 n-3	0.80 ± 0.20	0.95 ± 0.18	1.13 ± 0.38
20:0	0.60 ± 0.16	1.06 ± 0.22	0.88 ± 0.26
20:1 n-9	0.22 ± 0.22	0.57 ± 0.37	0.74 ± 0.47
20:2 n-6	0.98 ± 0.24	0.97 ± 0.13	1.27 ± 0.54
20:3 n-6	1.20 ± 0.27	1.71 ± 0.21	1.30 ± 0.30
20:4 n-6	7.18 ± 1.55	10.82 ± 1.84	9.43 ± 1.03
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.33 ± 0.33	0.62 ± 0.41	0.81 ± 0.52
20:5 n-3	0.39 ± 0.39	0.69 ± 0.44	0.79 ± 0.43
24:0	1.37 ± 0.93	0.69 ± 0.35	0.19 ± 0.19
22:4 n-6	0.26 ± 0.26	0.96 ± 0.61	0.92 ± 0.59
22:5 n-6	0.84 ± 0.23	1.56 ± 0.33	1.38 ± 0.23
24:1 n-9	0.49 ± 0.15	1.04 ± 0.26	0.93 ± 0.31
22:5 n-3	1.22 ± 0.30	1.54 ± 0.33	1.25 ± 0.26
22:6 n-3	4.16 ± 0.97	2.46 ± 0.23	2.43 ± 0.45
n-3	6.91 ± 1.69	6.27 ± 1.25	6.41 ± 1.76
n-6	18.78 ± 2.77	26.50 ± 2.92	22.72 ± 1.49
n-3 : n-6	0.33 ± 0.07	0.23 ± 0.03	0.28 ± 0.08

Table 3.7: Fatty acid profile in PC fraction of maternal liver, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.05 ± 0.04	0.06 ± 0.04	0.02 ± 0.02
14:0	0.39 ± 0.08	0.30 ± 0.02	0.26 ± 0.01
16:0	21.22 ± 0.80	21.10 ± 1.30	20.39 ± 0.80
16:1 n-7	1.74 ± 0.20	1.90 ± 0.13	1.66 ± 0.17
18:0	23.45 ± 0.59	22.73 ± 0.61	23.43 ± 1.13
18:1 n-9	10.45 ± 0.97	10.37 ± 1.16	10.20 ± 1.22
trans 18:1 n-9	1.73 ± 0.14^{a}	2.30 ± 0.18^{b}	1.63 ± 0.12^{a}
18:2 n-6	10.49 ± 0.28	10.95 ± 0.59	10.05 ± 0.43
18:3 n-6	0.79 ± 0.09	0.85 ± 0.08	0.83 ± 0.13
18:3 n-3	0.25 ± 0.07	0.27 ± 0.06	0.15 ± 0.02
20:0	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
20:1 n-9	0.28 ± 0.11	0.13 ± 0.04	0.07 ± 0.01
20:2 n-6	1.10 ± 0.16	0.93 ± 0.25	1.04 ± 0.23
20:4 n-6	12.53 ± 1.05^{a}	17.40 ± 1.60^{ab}	18.99 ± 1.37^{b}
22:0	0.00 ± 0.00	0.03 ± 0.02	0.01 ± 0.01
22:1 n-9 / 20:4 n-3	0.14 ± 0.05	0.10 ± 0.04	0.15 ± 0.03
20:5 n-3	2.18 ± 0.05^{b}	1.19 ± 0.17^{a}	1.10 ± 0.16^{a}
24:0	0.03 ± 0.03	0.00 ± 0.00	0.02 ± 0.02
22:4 n-6	0.61 ± 0.06	0.76 ± 0.06	0.84 ± 0.06
22:5 n-6	0.65 ± 0.08^{a}	1.25 ± 0.19^{b}	1.90 ± 0.12^{c}
24:1 n-9	0.09 ± 0.09	0.04 ± 0.04	0.00 ± 0.00
22:5 n-3	1.65 ± 0.15	1.37 ± 0.08	1.22 ± 0.13
22:6 n-3	7.57 ± 0.48^{b}	3.73 ± 0.50^{a}	3.67 ± 0.60^{a}
n-3	11.79 ± 0.54^{b}	6.66 ± 0.44^{a}	6.30 ± 0.52^{a}
n-6	28.77 ± 0.90^{a}	34.39 ± 1.31^{b}	36.00 ± 0.67^{b}
n-3 : n-6	0.41 ± 0.02^{b}	0.19 ± 0.01^{a}	0.17 ± 0.01^{a}

Table 3.8: Fatty acid profile in PE fraction of maternal liver, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.00 ± 0.00	0.20 ± 0.20	0.10 ± 0.10
14:0	0.17 ± 0.11	0.32 ± 0.09	0.36 ± 0.27
16:0	19.83 ± 0.64	19.08 ± 0.59	22.11 ± 3.05
16:1 n-7	1.40 ± 0.20	1.47 ± 0.13	2.30 ± 0.77
18:0	20.60 ± 0.91	21.57 ± 0.67	19.88± 2.90
18:1 n-9	6.68 ± 0.43	6.73 ± 0.69	12.19 ± 5.26
trans 18:1 n-9	3.50 ± 0.66	3.09 ± 0.46	2.20 ± 0.20
18:2 n-6	2.93 ± 1.35	3.35 ± 1.37	3.20 ± 1.05
18:3 n-6	3.04 ± 1.12	2.92 ± 1.62	2.45 ± 1.11
18:3 n-3	0.47 ± 0.19	0.34 ± 0.21	0.50 ± 0.15
20:0	0.34 ± 0.16	0.76 ± 0.20	0.25 ± 0.11
20:1 n-9	1.03 ± 0.35	0.28 ± 0.17	0.53 ± 0.05
20:2 n-6	1.58 ± 0.40	0.94 ± 0.17	0.71 ± 0.20
20:3 n-6	1.67 ± 0.08	1.65 ± 0.24	1.40 ± 0.25
20:4 n-6	15.49 ± 0.86	20.76 ± 0.65	19.08 ± 3.79
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.95 ± 0.28	1.21 ± 0.35	0.55 ± 0.16
20:5 n-3	1.82 ± 0.11	1.84 ± 0.29	1.03 ± 0.24
24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	1.83 ± 0.43	2.06 ± 0.11	1.80 ± 0.33
22:5 n-6	1.50 ± 0.21	1.71 ± 0.14	2.66 ± 0.54
24:1 n-9	0.52 ± 0.52	0.68 ± 0.48	0.00 ± 0.00
22:5 n-3	3.16 ± 0.21^{b}	2.85 ± 0.30^{ab}	1.87 ± 0.37^{a}
22:6 n-3	11.50 ± 1.46^{b}	6.20 ± 0.91^{a}	4.84 ± 1.24^{a}
n-3	17.90 ± 1.19^{b}	12.43 ± 0.66^{a}	8.79 ± 1.74^{a}
n-6	28.04 ± 0.94	33.40 ± 0.75	31.31 ± 5.03
n-3 : n-6	0.65 ± 0.06^{b}	0.37 ± 0.01^{a}	0.26 ± 0.03^{a}

Table 3.9: Fatty acid profile in TAG fraction of maternal liver, taken at weaning (3 weeks after birth of offspring). There were no statistical differences between any dietary groups. For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.04 ± 0.04	0.26 ± 0.19	0.00 ± 0.00
14:0	2.47 ± 0.44	2.42 ± 0.45	1.34 ± 0.18
16:0	35.51 ± 1.64	28.13 ± 2.41	30.96 ± 2.89
16:1 n-7	6.47 ± 0.42	6.45 ± 0.71	4.82 ± 0.72
18:0	4.01 ± 0.96	5.00 ± 1.34	8.60 ± 2.60
18:1 n-9	31.97 ± 4.02	29.79 ± 2.83	27.14 ± 4.00
trans 18:1 n-9	3.25 ± 1.38	6.02 ± 2.78	3.12 ± 0.61
18:2 n-6	7.24 ± 2.10	10.35 ± 2.08	4.04 ± 0.96
18:3 n-6	1.11 ± 0.41	0.63 ± 0.43	2.41 ± 0.96
18:3 n-3	0.36 ± 0.21	0.84 ± 0.34	0.71 ± 0.33
20:0	0.55 ± 0.48	0.41 ± 0.35	0.16 ± 0.16
20:1 n-9	0.47± 0.36	0.59 ± 0.33	0.25 ± 0.16
20:2 n-6	0.74 ± 0.41	0.24 ± 0.14	1.06 ± 0.24
20:3 n-6	0.92 ± 0.38	1.31 ± 0.72	2.26 ± 0.69
20:4 n-6	0.66 ± 0.36	1.38 ± 0.50	5.59 ± 3.10
22:0	0.70 ± 0.70	0.38 ± 0.38	0.09 ± 0.09
22:1 n-9 / 20:4 n-3	0.68 ± 0.41	0.96 ± 0.76	1.12 ± 0.69
20:5 n-3	0.03 ± 0.03	0.11 ± 0.11	0.33 ± 0.23
24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	0.48 ± 0.36	0.79 ± 0.34	0.72 ± 0.16
22:5 n-6	0.73 ± 0.61	0.95 ± 0.58	1.06 ± 0.64
24:1 n-9	0.44 ± 0.33	0.52 ± 0.30	0.72 ± 0.46
22:5 n-3	0.68 ± 0.52	1.24 ± 0.52	1.24 ± 0.54
22:6 n-3	0.48 ± 0.27	1.22 ± 0.52	2.26± 0.88
n-3	2.23 ± 0.68	4.38 ± 1.58	5.65 ± 1.28
n-6	11.88 ± 2.65	15.65 ± 3.78	17.14 ± 4.23
n-3 : n-6	0.23 ± 0.06	0.24 ± 0.04	0.36 ± 0.06

Table 3.10: Fatty acid profile in CE fraction of maternal spleen, taken at weaning (3 weeks after birth of offspring). There were no statistical differences between any dietary groups. For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	3.73 ± 1.11	1.94 ± 1.20	5.00 ± 1.08
12:0	1.63 ± 0.52	0.87 ± 0.50	2.09 ± 0.56
14:0	1.31 ± 0.21	0.84 ± 0.45	0.76 ± 0.26
16:0	11.76 ± 5.22	19.38 ± 7.05	6.04 ± 2.29
16:1 n-7	2.36 ± 1.12	3.18 ± 1.08	1.02 ± 0.27
18:0	5.20 ± 0.22	5.78 ± 0.67	5.07 ± 0.36
18:1 n-9	16.01 ± 4.50	17.96 ± 4.17	8.62 ± 0.42
trans 18:1 n-9	1.06 ± 0.56	3.56 ± 1.60	1.11 ± 0.68
18:2 n-6	9.27 ± 3.05	2.95 ± 1.82	6.49 ± 0.53
18:3 n-6	8.47 ± 2.46	12.30 ± 1.54	11.14 ± 1.06
18:3 n-3	0.20 ± 0.13	0.67 ± 0.30	0.00 ± 0.00
20:0	0.13 ± 0.10	1.29 ± 0.63	0.22 ± 0.14
20:1 n-9	0.73 ± 0.52	1.45 ± 0.86	0.00 ± 0.00
20:2 n-6	1.11 ± 0.30	0.76 ± 0.10	1.70 ± 0.32
20:3 n-6	1.24 ± 0.36	1.62 ± 0.41	1.50 ± 0.11
20:4 n-6	12.24 ± 3.48	8.26 ± 3.92	15.81 ± 1.36
22:0	1.03 ± 0.62	0.42 ± 0.26	0.90 ± 0.48
22:1 n-9 / 20:4 n-3	0.23 ± 0.17	1.44 ± 0.83	4.10 ± 2.67
20:5 n-3	11.18 ± 3.26	6.99 ± 4.01	11.74 ± 2.95
24:0	0.00 ± 0.00	0.00 ± 0.00	0.72 ± 0.72
22:4 n-6	0.05 ± 0.05	0.30 ± 0.19	0.74 ± 0.74
22:5 n-6	2.47 ± 1.11	1.34 ± 0.82	2.54 ± 0.86
24:1 n-9	0.47 ± 0.47	0.00 ± 0.00	1.78 ± 1.78
22:5 n-3	2.18 ± 0.65	1.97 ± 0.81	3.08 ± 0.48
22:6 n-3	5.95 ± 1.63	4.73 ± 1.88	7.82 ± 0.29
n-3	19.74 ± 5.07	15.80 ± 5.77	26.74 ± 0.76
n-6	34.83 ± 4.60	27.53 ± 6.80	39.92 ± 2.54
n-3 : n-6	0.50 ± 0.10	0.50 ± 0.09	0.68 ± 0.03

Table 3.11: Fatty acid profile in NEFA fraction of maternal spleen, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.0 0	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	1.82 ± 0.28	1.07 ± 0.37	1.87 ± 0.24
16:0	19.74 ± 1.32	18.20 ± 0.52	18.09 ± 0.35
16:1 n-7	2.69 ± 0.76	1.98 ± 0.27	1.71 ± 0.17
18:0	12.44 ± 1.88	14.43 ± 0.82	15.60 ± 0.40
18:1 n-9	18.67 ± 1.92	14.44 ± 1.19	14.46 ± 0.48
trans 18:1 n-9	3.60 ± 0.11	4.31 ± 1.00	3.11 ± 0.06
18:2 n-6	16.76 ± 1.50	11.68 ± 3.01	14.07 ± 0.75
18:3 n-6	0.00 ± 0.00	3.15 ± 3.15	0.00 ± 0.00
18:3 n-3	0.48 ± 0.31	0.33 ± 0.21	0.33 ± 0.22
20:0	1.10 ± 0.36	1.55 ± 0.43	0.52 ± 0.35
20:1 n-9	0.10 ± 0.10	0.18 ± 0.18	0.00 ± 0.00
20:2 n-6	1.18 ± 0.26	1.48 ± 0.25	1.37 ± 0.14
20:3 n-6	1.17 ± 0.20	1.63 ± 0.20	1.83 ± 0.24
20:4 n-6	10.59 ± 2.20	15.60 ± 1.33	16.24 ± 0.57
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.43 ± 0.28	0.40 ± 0.40	0.00 ± 0.00
20:5 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24:0	0.00 ± 0.00	0.36 ± 0.28	0.00 ± 0.00
22:4 n-6	0.00 ± 0.00	0.72 ± 0.72	0.00 ± 0.00
22:5 n-6	2.25 ± 0.43^{a}	3.48 ± 0.54^{ab}	4.58 ± 0.40^{b}
24:1 n-9	0.71 ± 0.38^{a}	1.04 ± 0.43^{ab}	1.95 ± 0.14^{b}
22:5 n-3	2.66 ± 0.42	1.92 ± 0.17	1.84 ± 0.28
22:6 n-3	3.61 ± 0.40^{b}	2.04 ± 0.31^{a}	2.43 ± 0.18^{a}
n-3	7.18 ± 0.59^{b}	4.70 ± 0.44^{a}	4.60 ± 0.27^{a}
n-6	31.96 ± 1.70^{a}	37.74 ± 1.75^{b}	38.09 ± 0.80^{b}
n-3 : n-6	0.22 ± 0.01^{b}	0.13 ± 0.02^{a}	0.12 ± 0.01^{a}

Table 3.12: Fatty acid profile in PC fraction of maternal spleen, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.26 ± 0.13	0.02 ± 0.02	0.13 ± 0.06
14:0	0.61 ± 0.10	0.78 ± 0.31	0.54 ± 0.11
16:0	38.49 ± 1.78	41.75 ± 1.68	42.65 ± 1.25
16:1 n-7	1.02 ± 0.09	0.75 ± 0.09	0.78 ± 0.04
18:0	11.77 ± 0.53	12.19 ± 0.31	12.79 ± 0.44
18:1 n-9	9.73 ± 1.41	7.03 ± 0.39	7.26 ± 0.28
trans 18:1 n-9	3.86 ± 0.14	3.78 ± 0.18	3.68 ± 0.12
18:2 n-6	13.42 ± 1.17^{b}	10.21 ± 1.07^{ab}	9.40 ± 0.75^{a}
18:3 n-6	0.50 ± 0.15	0.56 ± 0.19	0.37 ± 0.13
18:3 n-3	0.55 ± 0.17	0.29 ± 0.02	0.34 ± 0.10
20:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1 n-9	1.03 ± 0.31	1.66 ± 0.58	1.68 ± 0.32
20:2 n-6	0.47 ± 0.12	0.26 ± 0.11	0.24 ± 0.08
20:3 n-6	1.10 ± 0.06	0.93 ± 0.13	0.85 ± 0.06
20:4 n-6	10.02 ± 0.47	12.43 ± 1.38	12.86 ± 1.24
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.90 ± 0.13	0.85 ± 0.14	0.65 ± 0.11
20:5 n-3	0.72 ± 0.10	0.80 ± 0.35	0.59 ± 0.21
24:0	0.00 ± 0.00	0.24 ± 0.15	0.10 ± 0.10
22:4 n-6	1.38 ± 0.13^{a}	2.07 ± 0.17^{b}	2.12 ± 0.12^{b}
22:5 n-6	0.67 ± 0.09	0.96 ± 0.20	1.01 ± 0.24
24:1 n-9	0.66 ± 0.17	0.46 ± 0.25	0.35 ± 0.18
22:5 n-3	1.38 ± 0.07^{b}	1.10 ± 0.06^{ab}	0.88 ± 0.11^{a}
22:6 n-3	1.46 ± 0.27^{b}	0.88 ± 0.06^{ab}	0.73 ± 0.09^{a}
n-3	5.01 ± 0.28^{b}	3.91 ± 0.48^{ab}	3.19 ± 0.46^{a}
n-6	27.56 ± 1.02	27.42 ± 2.17	26.85 ± 1.48
n-3 : n-6	0.18 ± 0.01	0.15 ± 0.03	0.12 ± 0.02

Table 3.13: Fatty acid profile in PE fraction of maternal spleen, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	2.27 ± 0.25	2.21 ± 0.25	1.91 ± 0.20
16:0	10.60 ± 0.82	9.70 ± 0.48	9.14 ± 0.29
16:1 n-7	0.77 ± 0.12	1.06 ± 0.26	0.62 ± 0.15
18:0	15.50 ± 0.73	15.74 ± 0.49	16.48 ± 0.18
18:1 n-9	8.41 ± 1.59	6.93 ± 0.51	7.01 ± 0.17
trans 18:1 n-9	2.24 ± 0.56	2.46 ± 0.17	2.70 ± 0.35
18:2 n-6	7.39 ± 1.27	6.96 ± 0.73	5.67 ± 0.37
18:3 n-6	0.70 ± 0.13	0.78 ± 0.33	0.58 ± 0.16
18:3 n-3	0.46 ± 0.16	0.53 ± 0.14	0.51 ± 0.17
20:0	0.09 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
20:1 n-9	1.36 ± 0.35	1.13 ± 0.30	1.06 ± 0.17
20:2 n-6	1.14 ± 0.17	1.32 ± 0.26	0.82 ± 0.07
20:3 n-6	1.66 ± 0.23	2.38 ± 0.75	1.59 ± 0.31
20:4 n-6	27.10 ± 2.20^{a}	32.41 ± 1.84^{ab}	34.65 ± 1.53^{b}
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	1.37 ± 0.42	1.37 ± 0.30	1.26 ± 0.32
20:5 n-3	2.06 ± 0.26^{b}	0.54 ± 0.15^{a}	1.36 ± 0.30^{ab}
24:0	0.00 ± 0.00	0.21 ± 0.21	0.00 ± 0.00
22:4 n-6	4.08 ± 0.49^{a}	6.19 ± 0.57^{b}	6.74 ± 0.30^{b}
22:5 n-6	1.49 ± 0.19	1.94 ± 0.29	2.29 ± 0.25
24:1 n-9	0.41 ± 0.31	0.38 ± 0.31	0.58 ± 0.58
22:5 n-3	5.66 ± 0.35^{b}	3.51 ± 0.23^{a}	2.94 ± 0.15^{a}
22:6 n-3	5.26 ± 0.33^{b}	2.25 ± 0.25^{a}	2.09 ± 0.12^{a}
n-3	14.81 ± 0.74^{b}	8.20 ± 0.31^{a}	8.16 ± 0.70^{a}
n-6	43.55 ± 1.37^{a}	51.97 ± 0.44^{b}	52.34 ± 1.45^{b}
n-3 : n-6	0.34 ± 0.02^{b}	0.16 ± 0.01^{a}	0.16 ± 0.02^{a}

Table 3.14: Fatty acid profile in TAG fraction of maternal spleen, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.03
12:0	0.11 ± 0.05	0.53 ± 0.27	0.21 ± 0.11
14:0	1.90 ± 0.39	1.59 ± 0.25	2.03 ± 0.34
16:0	22.66 ± 3.48	22.25 ± 1.97	22.18 ± 1.76
16:1 n-7	5.62 ± 1.35	4.94 ± 1.04	5.35 ± 0.68
18:0	3.48 ± 0.26	3.71 ± 0.36	3.66 ± 0.32
18:1 n-9	17.99 ± 5.33	22.50 ± 2.52	24.71 ± 1.61
trans 18:1 n-9	5.23 ± 1.18	3.62 ± 0.51	3.62 ± 0.44
18:2 n-6	19.99 ± 3.56	23.91 ± 4.63	26.85 ± 2.27
18:3 n-6	2.37 ± 2.37	1.40 ± 1.29	0.05 ± 0.05
18:3 n-3	0.67 ± 0.31	1.11 ± 0.33	0.62 ± 0.22
20:0	1.03 ± 0.74	0.29 ± 0.29	0.91 ± 0.57
20:1 n-9	1.69 ± 0.37	0.83 ± 0.33	0.99 ± 0.20
20:2 n-6	1.61 ± 1.04	0.81 ± 0.20	1.42 ± 0.43
20:3 n-6	3.28 ± 3.08	2.29 ± 1.83	0.68 ± 0.32
20:4 n-6	1.00 ± 0.62	0.79 ± 0.42	0.51 ± 0.28
22:0	0.63 ± 0.41	0.38 ± 0.23	1.52 ± 1.09
22:1 n-9 / 20:4 n-3	4.48 ± 3.77	2.53 ± 2.53	0.00 ± 0.00
20:5 n-3	0.21 ± 0.14	0.00 ± 0.00	0.00 ± 0.00
24:0	0.00 ± 0.00	0.98 ± 0.98	0.67± 0.67
22:4 n-6	0.31 ± 0.18	1.09 ± 0.48	1.18 ± 0.40
22:5 n-6	1.03 ± 0.97	0.56 ± 0.41	1.44 ± 0.95
24:1 n-9	0.89 ± 0.83	0.68 ± 0.61	0.00 ± 0.00
22:5 n-3	1.49 ± 0.49	1.30 ± 0.39	0.37 ± 0.25
22:6 n-3	2.33 ± 0.66	1.94 ± 0.86	0.99 ± 0.43
n-3	9.19 ± 2.87	6.88 ± 2.60	1.98 ± 0.59
n-6	29.58 ± 3.50	30.84 ± 3.58	32.13 ± 2.50
n-3 : n-6	0.29 ± 0.05^{b}	0.22 ± 0.08^{ab}	0.06 ± 0.02^{a}

Table 3.15: Fatty acid profile in CE fraction of maternal thymus, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.78 ± 0.78	0.89 ± 0.89	0.00 ± 0.00
12:0	0.75 ± 0.66	0.62 ± 0.52	0.09 ± 0.03
14:0	3.17 ± 0.63	3.31 ± 0.71	3.47 ± 0.16
16:0	31.55 ± 6.76	32.85 ± 5.71	36.40 ± 1.25
16:1 n-7	5.04 ± 1.16	4.77 ± 1.01	6.84 ± 0.58
18:0	9.25 ± 1.08	8.34 ± 2.51	8.82 ± 0.77
18:1 n-9	19.87 ± 3.96^{ab}	16.12 ± 4.05^{a}	28.32 ± 1.49^{b}
trans 18:1 n-9	9.32 ± 3.88	13.46 ± 6.37	5.60 ± 1.53
18:2 n-6	2.44 ± 1.50	3.14 ± 1.94	0.65 ± 0.64
18:3 n-6	5.89 ± 2.16	6.42 ± 1.75	7.88 ± 1.99
18:3 n-3	0.03 ± 0.02	0.05 ± 0.04	0.01 ± 0.01
20:0	0.23 ± 0.06	0.26 ± 0.08	0.27 ± 0.05
20:1 n-9	0.83 ± 0.23^{b}	0.21 ± 0.10^{a}	0.28 ± 0.08^{a}
20:2 n-6	0.23 ± 0.13	0.44 ± 0.34	0.13 ± 0.01
20:3 n-6	0.08 ± 0.07	0.23 ± 0.11	0.12 ± 0.05
20:4 n-6	3.92 ± 3.77	3.27 ± 3.01	0.30 ± 0.14
22:0	0.22 ± 0.22	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.08 ± 0.05	0.10 ± 0.09	0.17 ± 0.13
20:5 n-3	3.55 ± 3.55	2.88 ± 2.88	0.00 ± 0.00
24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	0.00 ± 0.00^{a}	0.14 ± 0.06^{ab}	0.26 ± 0.08^{b}
22:5 n-6	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.02
24:1 n-9	0.08 ± 0.08	0.00 ± 0.00	0.05 ± 0.05
22:5 n-3	0.73 ± 0.57	0.43 ± 0.35	0.15 ± 0.10
22:6 n-3	1.94 ± 1.81	2.07 ± 1.99	0.15 ± 0.06
n-3	6.33 ± 5.91	5.52 ± 5.18	0.49 ± 0.28
n-6	12.57 ± 6.81	13.64 ± 5.45	9.37 ± 1.53
n-3 : n-6	0.21 ± 0.14	0.18 ± 0.14	0.06 ± 0.03

Table 3.16: Fatty acid profile in NEFA fraction of maternal thymus, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	1.53 ± 0.23	1.53 ± 0.28	1.34 ± 0.17
16:0	23.03 ± 1.16	22.73 ± 1.44	22.26 ± 0.61
16:1 n-7	5.10 ± 0.62	5.53 ± 0.61	5.79 ± 0.33
18:0	11.61 ± 0.63	11.59 ± 0.61	10.96 ± 0.26
18:1 n-9	26.22 ± 1.74	26.56 ± 2.18	28.11 ± 0.79
trans 18:1 n-9	3.67 ± 0.37	3.38 ± 0.30	3.48 ± 0.08
18:2 n-6	10.81 ± 0.77	12.15 ± 1.58	11.39 ± 0.91
18:3 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.27 ± 0.27
20:0	0.69 ± 0.23	0.82 ± 0.23	0.74 ± 0.23
20:1 n-9	0.34 ± 0.22	0.47 ± 0.47	0.00 ± 0.00
20:2 n-6	0.98 ± 0.20	1.00 ± 0.18	1.11 ± 0.18
20:3 n-6	1.55 ± 0.34	1.29 ± 0.20	1.22 ± 0.16
20:4 n-6	8.52 ± 1.77	8.59 ± 1.12	9.25 ± 0.49
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.49 ± 0.23	0.00 ± 0.00	0.00 ± 0.00
20:5 n-3	0.15 ± 0.15	0.00 ± 0.00	0.00 ± 0.00
24:0	0.29 ± 0.29	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:5 n-6	1.42 ± 0.21	1.97 ± 0.44	2.20 ± 0.37
24:1 n-9	0.08 ± 0.06^{a}	0.29 ± 0.21^{ab}	0.76 ± 0.15^{b}
22:5 n-3	1.42 ± 0.13^{b}	1.06 ± 0.29^{ab}	0.40 ± 0.18^{a}
22:6 n-3	2.10 ± 0.22^{b}	1.05 ± 0.20^{a}	0.71 ± 0.27^{a}
n-3	4.16 ± 0.33^{b}	2.11 ± 0.44^{a}	1.38 ± 0.58^{a}
n-6	23.28 ± 2.08	25.00 ± 2.11	25.18 ± 0.99
n-3 : n-6	0.18 ± 0.02^{b}	0.08 ± 0.02^{a}	0.06 ± 0.02^{a}

Table 3.17: Fatty acid profile in PC fraction of maternal thymus, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	1.39 ± 0.22	0.94 ± 0.13	1.20 ± 0.11
16:0	33.62 ± 0.67	35.92 ± 0.74	34.40 ± 0.95
16:1 n-7	1.98 ± 0.15^{b}	1.54 ± 0.06^{a}	1.51 ± 0.03^{a}
18:0	14.37 ± 1.08	15.32 ± 1.04	14.69 ± 0.68
18:1 n-9	13.41 ± 1.23	12.98 ± 0.76	12.84 ± 0.96
trans 18:1 n-9	5.20 ± 0.63	4.69 ± 0.39	4.51 ± 0.22
18:2 n-6	8.55 ± 0.56	8.42 ± 0.61	8.11 ± 0.24
18:3 n-6	0.26 ± 0.11	0.22 ± 0.09	0.15 ± 0.04
18:3 n-3	0.08 ± 0.08	0.08 ± 0.08	0.09 ± 0.07
20:0	0.37 ± 0.12	0.23 ± 0.12	0.17 ± 0.08
20:1 n-9	1.47 ± 0.60	1.21 ± 0.41	1.26 ± 0.26
20:2 n-6	1.25 ± 0.11	0.88 ± 0.23	1.24 ± 0.16
20:3 n-6	1.58 ± 0.13	1.49 ± 0.17	1.59 ± 0.25
20:4 n-6	9.65 ± 0.60^{a}	10.91 ± 0.68^{ab}	12.22 ± 0.65^{b}
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.80 ± 0.44	0.62 ± 0.21	0.53 ± 0.12
20:5 n-3	0.48 ± 0.16	0.17 ± 0.14	0.18 ± 0.12
24:0	0.07 ± 0.07	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	0.45 ± 0.34	0.85 ± 0.54	0.69 ± 0.32
22:5 n-6	1.63 ± 0.37	1.41 ± 0.52	1.74 ± 0.55
24:1 n-9	0.64 ± 0.22	0.55 ± 0.12	0.71 ± 0.15
22:5 n-3	0.99 ± 0.19	0.62 ± 0.21	0.81 ± 0.35
22:6 n-3	1.75 ± 0.34	0.96 ± 0.25	1.36 ± 0.56
n-3	4.10 ± 0.54	2.46 ± 0.22	2.97 ± 0.72
n-6	23.38 ± 0.49	24.17 ± 1.02	25.74 ± 0.81
n-3 : n-6	0.17 ± 0.02	0.10 ± 0.01	0.11 ± 0.02

Table 3.18: Fatty acid profile in PE fraction of maternal thymus, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12:0	0.18 ± 0.18	0.00 ± 0.00	0.00 ± 0.00
14:0	1.45 ± 0.34	1.29 ± 0.26	1.54 ± 0.29
16:0	11.10 ± 0.89	9.45 ± 0.78	10.72 ± 0.74
16:1 n-7	1.30 ± 0.35	0.99 ± 0.29	1.24 ± 0.22
18:0	17.13 ± 1.06	18.68 ± 0.27	18.56 ± 0.34
18:1 n-9	14.62 ± 1.23	11.11 ± 1.35	13.01 ± 0.71
trans 18:1 n-9	1.47 ± 0.67	4.38 ± 0.62	2.86 ± 0.78
18:2 n-6	5.28 ± 0.55	4.80 ± 0.76	4.51 ± 0.39
18:3 n-6	0.34 ± 0.10	0.59 ± 0.15	0.57 ± 0.16
18:3 n-3	0.56 ± 0.21	0.32 ± 0.14	0.38 ± 0.08
20:0	0.00 ± 0.00	0.47 ± 0.32	0.00 ± 0.00
20:1 n-9	1.66 ± 0.07	1.66 ± 0.56	1.67 ± 0.19
20:2 n-6	1.19 ± 0.23	0.83 ± 0.11	0.78 ± 0.09
20:3 n-6	1.70 ± 0.14	1.33 ± 0.11	1.50 ± 0.11
20:4 n-6	26.59 ± 2.87	30.74 ± 0.91	31.64 ± 1.56
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.96 ± 0.22	1.33 ± 0.18	0.97 ± 0.22
20:5 n-3	1.45 ± 0.16^{b}	0.85 ± 0.19^{ab}	0.79 ± 0.17^{a}
24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	3.46 ± 0.22	5.08 ± 0.73	4.45 ± 0.42
22:5 n-6	1.46 ± 0.31^{b}	2.49 ± 0.37^{a}	2.41 ± 0.17^{a}
24:1 n-9	0.13 ± 0.09	0.25 ± 0.25	0.00 ± 0.00
22:5 n-3	2.88 ± 0.28	1.49 ± 0.18	1.05 ± 0.13
22:6 n-3	5.09 ± 0.59^{b}	1.90 ± 0.08^{a}	1.34 ± 0.21^{a}
n-3	10.95 ± 1.14^{b}	5.89 ± 0.30^{a}	4.54 ± 0.35^{a}
n-6	40.02 ± 2.42	45.84 ± 1.54	45.87 ± 1.48
n-3 : n-6	0.28 ± 0.04^{b}	0.13 ± 0.01^{a}	0.10 ± 0.01^{a}

Table 3.19: Fatty acid profile in TAG fraction of maternal plasma, taken at weaning (3 weeks after birth of offspring). Values with different letters on the same line are statistically different from one another ($p < 0.05$). For all dietary groups, n = 6. Data shown as % abundance.

	Salmon oil	Soybean oil	Sunflower oil
8:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10:0	0.23 ± 0.15	0.12 ± 0.12	0.00 ± 0.00
12:0	0.29 ± 0.05	0.18 ± 0.06	0.22 ± 0.03
14:0	4.15 ± 0.37	3.65 ± 0.45	3.53 ± 0.17
16:0	31.99 ± 1.11	32.15 ± 1.45	31.48 ± 1.93
16:1 n-7	6.91 ± 0.51	6.04 ± 0.38	7.65 ± 0.67
18:0	4.77± 1.98	7.10 ± 1.87	1.92 ± 1.21
18:1 n-9	21.57± 5.20	18.33 ± 6.00	15.39 ± 4.29
trans 18:1 n-9	12.18 ± 6.64	13.59 ± 7.25	24.11 ± 7.10
18:2 n-6	8.16 ± 1.03	8.42 ± 2.39	8.97 ± 2.73
18:3 n-6	0.14 ± 0.12	3.17 ± 2.93	1.97 ± 1.69
18:3 n-3	0.28 ± 0.09	0.31 ± 0.03	0.17 ± 0.06
20:0	0.21 ± 0.17	0.12 ± 0.07	0.17 ± 0.05
20:1 n-9	1.56 ± 0.35^{b}	0.30 ± 0.05^{a}	0.34 ± 0.08^{a}
20:2 n-6	0.61 ± 0.40	0.90 ± 0.36	0.46 ± 0.34
20:3 n-6	0.29 ± 0.14	0.54 ± 0.30	0.30 ± 0.16
20:4 n-6	0.52 ± 0.31	0.30 ± 0.04	0.30 ± 0.04
22:0	0.09 ± 0.09	0.01 ± 0.01	0.00 ± 0.00
22:1 n-9 / 20:4 n-3	0.35 ± 0.16	0.29 ± 0.22	0.08 ± 0.08
20:5 n-3	0.53 ± 0.31	0.11 ± 0.11	0.00 ± 0.00
24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 n-6	0.72 ± 0.26	1.09 ± 0.52	1.13 ± 0.68
22:5 n-6	0.57 ± 0.23	0.95 ± 0.66	0.63 ± 0.37
24:1 n-9	0.90 ± 0.44	0.35 ± 0.26	0.04 ± 0.04
22:5 n-3	1.28 ± 0.24	0.83 ± 0.41	0.53 ± 0.31
22:6 n-3	1.72 ± 0.42	1.18 ± 0.59	0.63 ± 0.28
n-3	4.15 ± 0.86	2.72 ± 1.16	1.41 ± 0.62
n-6	11.01 ± 1.26	15.36 ± 0.46	13.74 ± 3.46
n-3 : n-6	0.41 ± 0.12^{b}	0.17 ± 0.07^{ab}	0.09 ± 0.03^{a}

Table 3.20: Fatty acid profile in CE fraction of offspring plasma, taken at various ages. There was no effect of diet on fatty acids, but there was an effect of time. Values with different letters on the same line are statistically different from one another ($p < 0.05$).

		Week 3	Week 6	Week 9	Week 12
12:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	Salmon oil	0.01 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Soybean oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:0	Salmon oil	0.15 ± 0.02^{b}	0.09 ± 0.00^{a}	0.11 ± 0.01^{a}	0.09 ± 0.00^{a}
	Soybean oil	0.13 ± 0.02	0.09 ± 0.00	0.11 ± 0.01	0.11 ± 0.01
	Sunflower oil	0.14 ± 0.01^{b}	0.09 ± 0.00^{a}	0.09 ± 0.01^{a}	0.11 ± 0.01^{a}
16:1 *n-7*	Salmon oil	0.02 ± 0.01^{a}	0.03 ± 0.00^{a}	0.08 ± 0.01^{b}	0.04 ± 0.01^{a}
	Soybean oil	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
	Sunflower oil	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}	0.09 ± 0.01^{b}	0.05 ± 0.01^{ab}
18:1 *n-9*	Salmon oil	0.12 ± 0.02^{b}	0.07 ± 0.00^{ab}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}
	Soybean oil	0.10 ± 0.01^{b}	0.07 ± 0.00^{ab}	0.07 ± 0.01^{ab}	0.05 ± 0.00^{a}
	Sunflower oil	0.11 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.03
trans 18:1 *n-9*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:2 *n-6*	Salmon oil	0.26 ± 0.01	0.23 ± 0.00	0.23 ± 0.01	0.24 ± 0.02
	Soybean oil	0.24 ± 0.00	0.24 ± 0.01	0.26 ± 0.01	0.27 ± 0.02
	Sunflower oil	0.23 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.24 ± 0.02
18:3 *n-6*	Salmon oil	0.01 ± 0.00^{c}	0.01 ± 0.00^{bc}	0.00 ± 0.00^{a}	0.00 ± 0.00^{ab}
	Soybean oil	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
18:*3 n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:2 *n-6*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:3 *n-6*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4 *n-6*	Salmon oil	0.29 ± 0.08^{a}	0.50 ± 0.01^{b}	0.44 ± 0.02^{ab}	0.49 ± 0.01^{b}
	Soybean oil	0.43 ± 0.02	0.47 ± 0.02	0.41 ± 0.03	0.45 ± 0.02
	Sunflower oil	0.29 ± 0.09^{a}	0.52 ± 0.01^{b}	0.44 ± 0.02^{ab}	0.44 ± 0.04^{ab}

		Week 3	Week 6	Week 9	Week 12
20:3 *n-9*	Salmon oil	0.05 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.13 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:*1 n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:5 *n-3*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:2 *n-9*	Salmon oil	0.01 ± 0.00[a]	0.02 ± 0.00[ab]	0.02 ± 0.00[a]	0.03 ± 0.00[b]
	Soybean oil	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01
	Sunflower oil	0.01 ± 0.00[a]	0.02 ± 0.00[ab]	0.03 ± 0.00[ab]	0.03 ± 0.00[b]
22:4 *n-6*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24:1 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:5 *n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:6 *n-3*	Salmon oil	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
	Soybean oil	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
	Sunflower oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
n-3	Salmon oil	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 001
	Soybean oil	0.02 ± 0.00	0.04 ± 0.00	0.03 ± 0.01	0.02 ± 0.01
	Sunflower oil	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
n-6	Salmon oil	0.57 ± 0.07	0.74 ± 0.01	0.67 ± 0.02	0.73 ± 0.02
	Soybean oil	0.68 ± 0.02	0.71 ± 0.01	0.67 ± 0.03	0.72 ± 0.02
	Sunflower oil	0.48 ± 0.10[a]	0.76 ± 0.00[b]	0.68 ± 0.02[ab]	0.67 ± 0.05[ab]
n-3 : *n-6*	Salmon oil	0.07 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
	Soybean oil	0.04 ± 0.01	0.06 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
	Sunflower oil	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02

Table 3.21: Fatty acid profile in NEFA fraction of offspring plasma, taken at various ages. There was no effect of diet on fatty acids, but there was an effect of time. Values with different letters on the same line are statistically different from one another ($p < 0.05$).

		Week 3	Week 6	Week 9	Week 12
12:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.01^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	Salmon oil	0.02 ± 0.00^{b}	0.00 ± 0.00^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}
	Soybean oil	0.02 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Sunflower oil	0.03 ± 0.00^{b}	0.00 ± 0.00^{a}	0.02 ± 0.00^{b}	0.03 ± 0.00^{b}
16:0	Salmon oil	0.22 ± 0.02^{a}	0.27 ± 0.01^{ab}	0.29 ± 0.01^{b}	0.29 ± 0.01^{b}
	Soybean oil	0.27 ± 0.03	0.25 ± 0.02	0.29 ± 0.01	0.30 ± 0.01
	Sunflower oil	0.19 ± 0.04^{a}	0.26 ± 0.01^{ab}	0.28 ± 0.01^{b}	0.29 ± 0.01^{b}
16:1 *n-7*	Salmon oil	0.01 ± 0.01^{a}	0.03 ± 0.01^{ab}	0.05 ± 0.01^{b}	0.04 ± 0.00^{b}
	Soybean oil	0.02 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
	Sunflower oil	0.06 ± 0.04	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.01
18:1 *n-9*	Salmon oil	0.10 ± 0.02^{a}	0.20 ± 0.02^{b}	0.15 ± 0.01^{ab}	0.09 ± 0.00^{a}
	Soybean oil	0.18 ± 0.04	0.17 ± 0.02	0.16 ± 0.03	0.09 ± 0.01
	Sunflower oil	0.12 ± 0.02^{a}	0.21 ± 0.01^{b}	0.13 ± 0.01^{a}	0.11 ± 0.01^{a}
trans 18:1 *n-9*	Salmon oil	0.02 ± 0.01^{a}	0.15 ± 0.01^{b}	0.04 ± 0.02^{a}	0.02 ± 0.00^{a}
	Soybean oil	0.07 ± 0.03^{ab}	0.14 ± 0.01^{b}	0.05 ± 0.02^{a}	0.02 ± 0.00^{a}
	Sunflower oil	0.05 ± 0.02^{a}	0.14 ± 0.02^{b}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}
18:2 *n-6*	Salmon oil	0.11 ± 0.02^{a}	0.19 ± 0.01^{b}	0.18 ± 0.01^{b}	0.18 ± 0.01^{b}
	Soybean oil	0.13 ± 0.02^{a}	0.18 ± 0.01^{b}	0.18 ± 0.00^{ab}	0.18 ± 0.01^{ab}
	Sunflower oil	0.10 ± 0.01^{a}	0.19 ± 0.01^{b}	0.16 ± 0.01^{b}	0.17 ± 0.01^{b}
18:3 *n-6*	Salmon oil	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
	Soybean oil	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
18:3 *n-3*	Salmon oil	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
20:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1 *n-9*	Salmon oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
	Soybean oil	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
20:2 *n-6*	Salmon oil	0.23 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
	Soybean oil	0.09 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.19 ± 0.08^{b}	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
20:3 *n-6*	Salmon oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4 *n-6*	Salmon oil	0.05 ± 0.01^{a}	0.09 ± 0.01^{ab}	0.07 ± 0.01^{ab}	0.10 ± 0.01^{b}
	Soybean oil	0.04 ± 0.01	0.07 ± 0.00	0.08 ± 0.00	0.08 ± 0.02
	Sunflower oil	0.04 ± 0.01^{a}	0.09 ± 0.01^{b}	0.08 ± 0.01^{b}	0.09 ± 0.01^{b}

		Week 3	Week 6	Week 9	Week 12
20:3 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.02
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
	Soybean oil	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:5 *n-3*	Salmon oil	0.00 ± 0.00[a]	0.01 ± 0.00[b]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:2 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.00 ± 0.00
22:4 *n-6*	Salmon oil	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.00[ab]	0.04 ± 0.02[b]	0.00 ± 0.00[a]	0.00 ± 0.00[ab]
	Sunflower oil	0.01 ± 0.00[ab]	0.02 ± 0.01[b]	0.00 ± 0.00[a]	0.01 ± 0.00[ab]
24:0	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24:1 *n-9*	Salmon oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:5 *n-3*	Salmon oil	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Soybean oil	0.01 ± 0.00[a]	0.03 ± 0.01[b]	0.02 ± 0.00[ab]	0.02 ± 0.00[ab]
	Sunflower oil	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
22:6 *n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
n-3	Salmon oil	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	Soybean oil	0.03 ± 0.01[a]	0.06 ± 0.00[b]	0.04 ± 0.00[ab]	0.04 ± 0.00[ab]
	Sunflower oil	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
n-6	Salmon oil	0.43 ± 0.06[b]	0.31 ± 0.02[ab]	0.25 ± 0.02[a]	0.29 ± 0.02[ab]
	Soybean oil	0.29 ± 0.07	0.32 ± 0.02	0.26 ± 0.01	0.27 ± 0.02
	Sunflower oil	0.41 ± 0.06[b]	0.31 ± 0.01[ab]	0.26 ± 0.01[a]	0.28 ± 0.01[a]
n-3 : *n-6*	Salmon oil	0.08 ± 0.01[a]	0.13 ± 0.02[ab]	0.15 ± 0.02[b]	0.15 ± 0.01[b]
	Soybean oil	0.12 ± 0.05	0.18 ± 0.03	0.14 ± 0.01	0.14 ± 0.01
	Sunflower oil	0.09 ± 0.03	0.13 ± 0.03	0.14 ± 0.02	0.16 ± 0.02

Table 3.22: Fatty acid profile in PC fraction of offspring plasma, taken at various ages. There was no effect of diet on fatty acids, but there was an effect of time. Values with different letters on the same line are statistically different from one another ($p < 0.05$).

		Week 3	Week 6	Week 9	Week 12
12:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	Salmon oil	0.01 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
16:0	Salmon oil	0.27 ± 0.01^{b}	0.25 ± 0.01^{ab}	0.24 ± 0.00^{a}	0.24 ± 0.01^{ab}
	Soybean oil	0.29 ± 0.01^{b}	0.25 ± 0.01^{a}	0.24 ± 0.00^{a}	0.25 ± 0.01^{a}
	Sunflower oil	0.31 ± 0.04	0.26 ± 0.00	0.23 ± 0.01	0.25 ± 0.01
16:1 *n-7*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.03 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:1 *n-9*	Salmon oil	0.11 ± 0.03^{a}	0.22 ± 0.01^{b}	0.07 ± 0.03^{a}	0.04 ± 0.01^{a}
	Soybean oil	0.12 ± 0.04^{ab}	0.22 ± 0.02^{b}	0.08 ± 0.04^{a}	0.04 ± 0.00^{a}
	Sunflower oil	0.09 ± 0.04^{a}	0.23 ± 0.02^{b}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}
trans 18:1 *n-9*	Salmon oil	0.04 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	Soybean oil	0.04 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
	Sunflower oil	0.03 ± 0.02	0.06 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
18:2 *n-6*	Salmon oil	0.24 ± 0.02	0.22 ± 0.01	0.23 ± 0.01	0.23 ± 0.02
	Soybean oil	0.23 ± 0.01	0.23 ± 0.02	0.25 ± 0.01	0.25 ± 0.01
	Sunflower oil	0.21 ± 0.03	0.22 ± 0.02	0.22 ± 0.00	0.23 ± 0.01
18:3 *n-6*	Salmon oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:3 *n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
20:2 *n-6*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:3 *n-6*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:4 *n-6*	Salmon oil	0.13 ± 0.01^{a}	0.17 ± 0.01^{b}	0.17 ± 0.00^{b}	0.17 ± 0.01^{b}
	Soybean oil	0.14 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.00
	Sunflower oil	0.13 ± 0.02	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01

		Week 3	Week 6	Week 9	Week 12
20:3 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:5 *n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00[a]	0.01 ± 0.00[b]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
22:2 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 *n-6*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24:0	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24:1 *n-9*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:5 *n-3*	Salmon oil	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
	Soybean oil	0.02 ± 0.01[a]	0.03 ± 0.00[ab]	0.04 ± 0.00[b]	0.04 ± 0.00[b]
	Sunflower oil	0.02 ± 0.00[a]	0.03 ± 0.00[a]	0.04 ± 0.00[b]	0.04 ± 0.00[b]
22:6 *n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
n-3	Salmon oil	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
	Soybean oil	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
	Sunflower oil	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
n-6	Salmon oil	0.41 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
	Soybean oil	0.40 ± 0.01	0.41 ± 0.01	0.42 ± 0.01	0.42 ± 0.01
	Sunflower oil	0.37 ± 0.04	0.40 ± 0.01	0.41 ± 0.01	0.40 ± 0.01
n-3 : *n-6*	Salmon oil	0.12 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
	Soybean oil	0.07 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
	Sunflower oil	0.09 ± 0.02	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.01

Table 3.23: Fatty acid profile in TAG fraction of offspring plasma, taken at various ages. There was no effect of diet on fatty acids, but there was an effect of time. Values with different letters on the same line are statistically different from one another ($p < 0.05$).

		Week 3	Week 6	Week 9	Week 12
12:0	Salmon oil	0.05 ± 0.02^{b}	0.01 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Soybean oil	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.04 ± 0.02^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
14:0	Salmon oil	0.06 ± 0.02	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.03 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.06 ± 0.03^{b}	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
16:0	Salmon oil	0.27 ± 0.03	0.25 ± 0.01	0.25 ± 0.01	0.26 ± 0.00
	Soybean oil	0.27 ± 0.02	0.16 ± 0.08	0.24 ± 0.00	0.26 ± 0.01
	Sunflower oil	0.28 ± 0.02	0.24 ± 0.01	0.23 ± 0.01	0.26 ± 0.01
16:1 *n-7*	Salmon oil	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00
	Soybean oil	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	Sunflower oil	0.02 ± 0.00	0.04 ± 0.00	0.10 ± 0.06	0.05 ± 0.01
18:1 *n-9*	Salmon oil	0.22 ± 0.02	0.24 ± 0.02	0.22 ± 0.01	0.21 ± 0.01
	Soybean oil	0.25 ± 0.03	0.28 ± 0.04	0.23 ± 0.02	0.19 ± 0.01
	Sunflower oil	0.23 ± 0.02	0.26 ± 0.01	0.21 ± 0.02	0.22 ± 0.01
trans 18:1 *n-9*	Salmon oil	0.04 ± 0.02	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.00
	Soybean oil	0.02 ± 0.01^{a}	0.05 ± 0.01^{b}	0.05 ± 0.00^{ab}	0.04 ± 0.00^{ab}
	Sunflower oil	0.03 ± 0.01^{a}	0.05 ± 0.00^{b}	0.04 ± 0.00^{b}	0.05 ± 0.00^{b}
18:2 *n-6*	Salmon oil	0.18 ± 0.03^{a}	0.28 ± 0.03^{b}	0.29 ± 0.01^{b}	0.28 ± 0.01^{b}
	Soybean oil	0.25 ± 0.02	0.31 ± 0.04	0.29 ± 0.02	0.29 ± 0.01
	Sunflower oil	0.20 ± 0.02^{a}	0.28 ± 0.01^{b}	0.25 ± 0.02^{ab}	0.26 ± 0.02^{ab}
18:3 *n-6*	Salmon oil	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:3 *n-3*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Soybean oil	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
	Sunflower oil	0.01 ± 0.00^{a}	0.02 ± 0.00^{ab}	0.02 ± 0.00^{ab}	0.02 ± 0.00^{b}
20:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1 *n-9*	Salmon oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
20:2 *n-6*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
20:3 *n-6*	Salmon oil	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4 *n-6*	Salmon oil	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.00
	Soybean oil	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.01	0.06 ± 0.01
	Sunflower oil	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01

		Week 3	Week 6	Week 9	Week 12
20:3 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 *n-9*	Salmon oil	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:5 *n-3*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:2 *n-9*	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
22:4 *n-6*	Salmon oil	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
24:0	Salmon oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Soybean oil	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
24:1 *n-9*	Salmon oil	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:5 *n-3*	Salmon oil	0.02 ± 0.00	0.03 ± 0.02	0.01 ± 0.00	0.02 ± 0.00
	Soybean oil	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Sunflower oil	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
22:6 *n-3*	Salmon oil	0.00 ± 0.00	0.04 ± 0.03	0.01 ± 0.01	0.00 ± 0.00
	Soybean oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sunflower oil	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
n-3	Salmon oil	0.04 ± 0.01	0.08 ± 0.04	0.05 ± 0.01	0.04 ± 0.00
	Soybean oil	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
	Sunflower oil	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.00
n-6	Salmon oil	0.26 ± 0.03^{a}	0.33 ± 0.02^{ab}	0.35 ± 0.01^{b}	0.34 ± 0.01^{ab}
	Soybean oil	0.32 ± 0.03	0.39 ± 0.04	0.36 ± 0.02	0.36 ± 0.02
	Sunflower oil	0.27 ± 0.04	0.34 ± 0.01	0.33 ± 0.03	0.34 ± 0.02
n-3 : *n-6*	Salmon oil	0.16 ± 0.04	0.23 ± 0.12	0.14 ± 0.02	0.12 ± 0.01
	Soybean oil	0.08 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
	Sunflower oil	0.09 ± 0.01	0.12 ± 0.02	0.12 ± 0.01	0.13 ± 0.01

Appendix Four – Data from Effect of Early Exposure to Fatty Acids on Allergic Sensitisation Study (Chapter 5).

Table 4-1: Fatty acid composition of diets. Data is shown as percentage abundance.

	Control	ALA-rich	LA-rich	"Western" diet
14:0	0.09	0.15	0.13	2.78
16:0	10.92	7.57	10.69	22.31
18:0	3.15	3.22	2.05	10.12
20:0	0.33	0.28	0.42	0.32
22:0	0.50	0.22	0.19	0.18
24:0	0.17	0.14	0.18	0.07
18:3 *n-3*	5.01	25.03	1.78	2.19
18:4 *n-3*	0.08	0.05	0.05	0.05
22:5 *n-3*	0.00	0.00	0.00	0.05
22:6 *n-3*	0.00	0.00	0.03	0.03
18:2 *n-6*	53.51	32.56	54.03	11.62
18:3 *n-6*	0.40	2.61	0.18	0.06
20:2 *n-6*	0.13	0.14	0.18	0.54
20:3 *n-6*	0.00	0.00	0.07	0.04
20:4 *n-6*	0.06	0.03	0.00	0.15
22:4 *n-6*	0.00	0.00	0.00	0.04
16:1 *n-7*	0.09	0.09	0.10	1.07
18:1 *n-7*	1.64	0.63	0.54	1.97
18:1 *n-9*	22.29	23.10	27.79	38.37
20:1 *n-9*	0.62	2.88	0.42	0.73
20:3 *n-9*	0.07	0.00	0.11	0.00
22:1 *n-9*	0.00	0.03	0.00	0.04
24:1 *n-9*	0.04	0.02	0.00	0.03
n-3	5.20	25.31	1.88	2.37
n-6	54.66	36.06	55.23	12.58
n-3 : *n-6*	9.51	70.17	3.41	18.83

Table 4.2: Fatty acid content of erythrocytes, taken at weaning, of mice fed the experimental diets during gestation and / or lactation. Data are mean +/- SEM % of total fatty acids. Values with different letters denote differences in fatty acid abundance between dietary groups.

	Control	AG	AGL	AL	LG	LGL	LL
14:0	0.20 ± 0.02	0.16 ± 0.01	0.20 ± 0.01	0.20 ± 0.03	0.19 ± 0.01	0.18 ± 0.01	0.21 ± 0.03
16:0	46.00 ± 1.43	43.42 ± 1.38	42.38 ± 2.51	44.38 ± 1.64	44.51 ± 1.85	44.28 ± 1.15	45.02 ± 1.53
18:0	14.92 ± 1.45	14.49 ± 0.88	14.15 ± 1.13	14.50 ± 0.91	15.47 ± 1.11	13.73 ± 0.86	14.02 ± 0.95
20:0	0.37 ± 0.03	0.32 ± 0.01	0.29 ± 0.01	0.34 ± 0.04	0.35 ± 0.01	0.36 ± 0.04	0.35 ± 0.02
22:0	1.14 ± 0.04^{ab}	1.07 ± 0.01^{ab}	0.95 ± 0.06^{a}	1.17 ± 0.08^{b}	1.12 ± 0.05^{ab}	1.00 ± 0.01^{ab}	1.09 ± 0.02^{ab}
24:0	1.53 ± 0.07^{ab}	1.45 ± 0.03^{ab}	1.56 ± 0.06^{ab}	1.76 ± 0.15^{b}	1.49 ± 0.05^{ab}	1.30 ± 0.04^{a}	1.37 ± 0.05^{a}
18:3 *n-3*	0.09 ± 0.01^{a}	0.20 ± 0.09^{a}	0.66 ± 0.18^{b}	0.39 ± 0.12^{ab}	0.10 ± 0.03^{a}	0.06 ± 0.01^{a}	0.04 ± 0.01^{a}
18:4 *n-3*	0.04 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.08 ± 0.05	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.02
20:5 *n-3*	0.10 ± 0.01^{a}	0.16 ± 0.04^{ab}	1.01 ± 0.08^{b}	1.08 ± 0.41^{b}	0.08 ± 0.01^{a}	0.07 ± 0.02^{a}	0.06 ± 0.02^{a}
22:5 *n-3*	0.32 ± 0.05^{ab}	0.50 ± 0.04^{b}	1.28 ± 0.11^{c}	1.02 ± 0.10^{c}	0.30 ± 0.04^{ab}	0.20 ± 0.03^{a}	0.19 ± 0.03^{a}
22:6 *n-3*	1.15 ± 0.10^{a}	1.60 ± 0.09^{a}	2.09 ± 0.08^{b}	1.80 ± 0.09^{ab}	1.13 ± 0.09^{a}	1.06 ± 0.09^{a}	1.16 ± 0.09^{a}
18:2 *n-6*	6.84 ± 0.44	8.22 ± 0.66	8.89 ± 1.15	7.20 ± 0.60	7.72 ± 1.12	7.70 ± 0.70	6.88 ± 0.79
18:3 *n-6*	0.15 ± 0.02	0.12 ± 0.00	0.10 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.16 ± 0.04	0.15 ± 0.03
20:2 *n-6*	0.46 ± 0.08	0.55 ± 0.08	0.38 ± 0.06	0.39 ± 0.06	0.42 ± 0.07	0.65 ± 0.09	0.59 ± 0.08
20:3 *n-6*	0.64 ± 0.07	0.77 ± 0.06	0.61 ± 0.04	0.58 ± 0.03	0.60 ± 0.05	0.68 ± 0.07	0.64 ± 0.06
20:4 *n-6*	6.37 ± 0.34^{ab}	7.35 ± 0.44^{b}	6.20 ± 0.44^{ab}	5.76 ± 0.18^{a}	6.69 ± 0.17^{ab}	7.78 ± 0.22^{b}	7.69 ± 0.45^{b}
22:4 *n-6*	0.86 ± 0.08^{b}	0.86 ± 0.07^{b}	0.35 ± 0.02^{a}	0.43 ± 0.04^{a}	0.89 ± 0.06^{bc}	1.23 ± 0.09^{d}	1.18 ± 0.07^{cd}

	Control	AG	AGL	AL	LG	LGL	LL
22:5 *n-6*	0.34 ± 0.05^{bc}	0.22 ± 0.02^{ab}	0.09 ± 0.02^{a}	0.16 ± 0.02^{a}	0.38 ± 0.05^{c}	0.78 ± 0.03^{d}	0.66 ± 0.01^{d}
16:1 *n-7*	0.72 ± 0.04	0.74 ± 0.06	0.73 ± 0.05	0.58 ± 0.07	0.70 ± 0.04	0.63 ± 0.03	0.61 ± 0.03
18:1 *n-7*	2.12 ± 0.01^{b}	2.01 ± 0.05^{b}	1.58 ± 0.08^{a}	1.56 ± 0.08^{a}	2.16 ± 0.04^{b}	1.62 ± 0.05^{a}	1.64 ± 0.04^{a}
18:1 *n-9*	11.57 ± 0.16	11.99 ± 0.28	12.89 ± 0.53	12.22 ± 0.36	11.98 ± 0.42	12.49 ± 0.42	12.32 ± 0.26
20:1 *n-9*	0.33 ± 0.02	0.32 ± 0.02	0.24 ± 0.02	0.30 ± 0.03	0.31 ± 0.01	0.33 ± 0.01	0.33 ± 0.02
20:3 *n-9*	0.09 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.08 ± 0.03	0.09 ± 0.02	0.06 ± 0.01	0.08 ± 0.02
22:1 *n-9*	0.90 ± 0.12	0.76 ± 0.08	0.60 ± 0.07	0.88 ± 0.12	0.78 ± 0.11	0.85 ± 0.10	0.94 ± 0.29
24:1 *n-9*	1.07 ± 0.06	1.02 ± 0.06	0.89 ± 0.08	1.13 ± 0.09	1.04 ± 0.08	1.10 ± 0.06	1.10 ± 0.06
n-3	1.70 ± 0.15^{a}	2.50 ± 0.19^{a}	5.07 ± 0.34^{b}	4.37 ± 0.45^{b}	1.64 ± 0.12^{a}	1.41 ± 0.12^{a}	1.50 ± 0.13^{a}
n-6	15.65 ± 0.77^{ab}	18.09 ± 0.90^{ab}	16.61 ± 1.49^{ab}	14.66 ± 0.65^{a}	16.83 ± 1.01^{ab}	18.97 ± 0.43^{b}	17.78 ± 1.06^{ab}
n-3 : n-6	0.11 ± 0.01^{a}	0.14 ± 0.01^{a}	0.31 ± 0.02^{b}	0.30 ± 0.03^{b}	0.10 ± 0.01^{a}	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}

Table 4.3: Fatty acid content of red blood cells, taken at euthanisation (age 78 days), of mice fed the experimental diets during gestation and / or lactation. Data are mean +/- SEM % of total fatty acids. Values with different letters denote differences in fatty acid abundance between dietary groups.

	Control	AG	AGL	AL	LG	LGL	LL
14:0	0.19 ± 0.02	0.18 ± 0.01	0.20 ± 0.04	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
16:0	32.39 ± 0.19	32.98 ± 0.18	33.23 ± 0.42	33.00 ± 0.30	33.01 ± 0.26	32.69 ± 0.17	32.95 ± 0.25
18:0	12.32 ± 0.07	12.36 ± 0.18	12.84 ± 0.08	12.76 ± 0.09	12.64 ± 0.13	12.94 ± 0.17	12.56 ± 0.10
20:0	0.35 ± 0.01	0.33 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.33 ± 0.02	0.34 ± 0.01
22:0	0.84 ± 0.01	0.82 ± 0.02	0.87 ± 0.01	0.85 ± 0.02	0.86 ± 0.03	0.85 ± 0.02	0.87 ± 0.02
24:0	0.99 ± 0.01	0.95 ± 0.03	1.03 ± 0.02	1.03 ± 0.04	1.00 ± 0.04	1.00 ± 0.02	1.02 ± 0.01
18:3 n-3	0.09 ± 0.00	0.09 ± 0.01	0.11 ± 0.03	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.01	0.09 ± 0.00
18:4 n-3	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.00	0.04 ± 0.01
20:5 n-3	0.27 ± 0.02	0.26 ± 0.01	0.28 ± 0.01	0.32 ± 0.02	0.24 ± 0.02	0.28 ± 0.03	0.24 ± 0.01
22:5 n-3	0.57 ± 0.03	0.54 ± 0.01	0.59 ± 0.02	0.61 ± 0.02	0.53 ± 0.02	0.51 ± 0.03	0.53 ± 0.02
22:6 n-3	3.83 ± 0.05	3.85 ± 0.07	3.95 ± 0.11	3.82 ± 0.38	3.76 ± 0.09	3.91 ± 0.14	3.71 ± 0.07
18:2 n-6	7.78 ± 0.31	7.34 ± 0.14	8.08 ± 0.16	8.09 ± 0.10	7.79 ± 0.18	7.52 ± 0.18	7.70 ± 0.13
18:3 n-6	0.08 ± 0.01	0.09 ± 0.02	0.11 ± 0.05	0.08 ± 0.02	0.08 ± 0.02	0.06 ± 0.00	0.06 ± 0.00
20:2 n-6	0.38 ± 0.04	0.46 ± 0.04	0.41 ± 0.06	0.47 ± 0.03	0.47 ± 0.05	0.49 ± 0.04	0.42 ± 0.03
20:3 n-6	1.20 ± 0.01	1.14 ± 0.02	1.15 ± 0.05	1.18 ± 0.02	1.12 ± 0.01	1.15 ± 0.03	1.14 ± 0.02
20:4 n-6	14.51 ± 0.11	14.66 ± 0.14	13.51 ± 0.13	14.46 ± 0.08	14.53 ± 0.14	14.77 ± 0.29	14.66 ± 0.23
22:4 n-6	1.26 ± 0.03^{bc}	1.27 ± 0.02^{bc}	1.12 ± 0.01^{a}	1.16 ± 0.01^{ab}	1.27 ± 0.02^{bc}	1.23 ± 0.03^{abc}	1.29 ± 0.03^{c}

	Control	AG	AGL	AL	LG	LGL	LL
22:5 n-6	0.90 ± 0.02[b]	0.87 ± 0.02[b]	0.62 ± 0.02[a]	0.64 ± 0.02[a]	0.87 ± 0.04[b]	0.90 ± 0.06[b]	0.94 ± 0.02[b]
16:1 n-7	0.45 ± 0.02	0.48 ± 0.03	0.44 ± 0.03	0.40 ± 0.02	0.41 ± 0.01	0.41 ± 0.01	0.40 ± 0.01
18:1 n-7	1.69 ± 0.07	1.75 ± 0.08	1.56 ± 0.03	1.73 ± 0.19	1.57 ± 0.02	1.66 ± 0.04	1.57 ± 0.03
18:1 n-9	16.27 ± 0.10	16.25 ± 0.20	16.22 ± 0.08	15.75 ± 0.37	16.01 ± 0.15	15.84 ± 0.13	15.88 ± 0.13
20:1 n-9	0.70 ± 0.02	0.66 ± 0.02	0.62 ± 0.03	0.64 ± 0.03	0.63 ± 0.02	0.60 ± 0.04	0.68 ± 0.02
20:3 n-9	0.22 ± 0.06	0.14 ± 0.06	0.13 ± 0.06	0.06 ± 0.04	0.04 ± 0.04	0.06 ± 0.04	0.12 ± 0.04
22:1 n-9	0.41 ± 0.06	0.30 ± 0.03	0.34 ± 0.03	0.32 ± 0.02	0.34 ± 0.02	0.34 ± 0.01	0.38 ± 0.02
24:1 n-9	1.54 ± 0.03	1.46 ± 0.03	1.45 ± 0.04	1.30 ± 0.21	1.49 ± 0.03	1.46 ± 0.04	1.56 ± 0.02
n-3	4.79 ± 0.09	4.78 ± 0.09	4.98 ± 0.10	4.88 ± 0.39	4.66 ± 0.13	4.84 ± 0.15	4.61 ± 0.11
n-6	26.09 ± 0.28[b]	25.84 ± 0.28[ab]	25.00 ± 0.09[a]	26.09 ± 0.16[b]	26.12 ± 0.13[b]	26.11 ± 0.21[b]	26.20 ± 0.18[b]
n-3 : n-6	0.18 ± 0.00	0.18 ± 0.00	0.20 ± 0.00	0.19 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	0.18 ± 0.00

Appendix Five – Data from the Effect of Intra-Uterine Growth Retardation on the Immune System of Offspring in Two Generations Study (Chapter 6)

Table 5-1: Fatty acid composition of diets. Data is shown as percentage abundance.

	Maintenance diet	Lactation Diet	Control	Protein reduced	Control + folate
12:0	0.43	0.00	0.25	0.29	0.36
14:0	0.39	0.40	0.43	0.28	0.36
16:0	7.75	6.00	9.29	7.12	4.54
16:1 n-7	0.18	0.22	0.14	0.00	0.49
18:0	3.01	3.47	3.73	2.61	2.46
18:1 n-9	4.04	4.60	8.47	6.89	3.01
trans 18:1 n-9	0.46	1.00	0.81	0.73	0.29
18:2 n-6	17.10	13.09	23.25	19.37	9.49
18:3 n-6	0.53	0.60	0.35	0.00	0.09
18:3 n-3	2.11	2.05	3.44	2.88	1.45
20:0	0.00	0.00	0.00	0.00	0.00
20:1 n-9	1.00	0.15	0.29	0.12	0.24
20:2 n-6	59.98	65.98	46.95	57.57	75.65
20:3 n-6	0.16	0.19	0.38	0.00	0.00
20:4 n-6	0.45	0.26	0.37	0.28	0.13
22:0	0.27	0.56	0.33	0.14	0.36
22:1 n-9	0.00	0.00	0.00	0.00	0.00
20:5 n-3	0.91	0.57	0.41	0.50	0.05
24:0	0.00	0.00	0.00	0.00	0.00
22:4 n-6	0.58	0.33	0.66	0.00	0.00
22:5 n-6	0.00	0.00	0.00	0.21	0.19
24:1 n-9	0.21	0.00	0.00	0.28	0.19
22:5 n-6	0.45	0.52	0.18	0.73	0.46
22:6 n-3	0.00	0.00	0.25	0.00	0.17
n-3	3.50	3.10	4.30	4.10	2.10
n-6	78.80	80.40	72.00	77.40	85.60
n-3 : n-6	0.00	0.00	0.10	0.10	0.00