The Importance of Long Chain Polyunsaturated Fatty Acids for Fetal Immune System Maturation and Function

Therishnee Moodley

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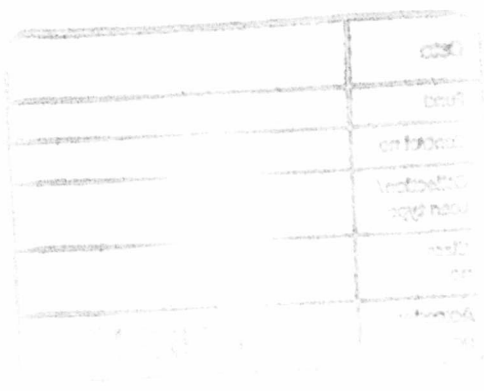

Abstract

Long chain polyunsaturated fatty acids (LCPUFAs) are important structural components in the cell membrane and a deficiency in these fatty acids have been linked to clinical complications. In addition, omega 3 and omega 6 LCPUFAs are biologically active substrates for the synthesis of regulators of inflammatory processes. Preterm neonates and uninfected neonates born to HIV-infected women (DNI) are susceptible to immune complications. These babies may have deficiencies in LCPUFAs, but the impact of this loss on maturation of the immune system is unknown. Cord blood mononuclear cell (CBMC) membrane fatty acid content was examined by gas chromatography (GC), and immunological profiles as defined by flow cytometry were compared between healthy term, preterm and DNI neonates as an indicator of immune deficiencies. This was the first study to examine the lipid profile of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions of CBMC membranes from preterm and DNI neonates. The LCPUFA composition of CBMCs was dominated by arachidonic acid (AA, 20:4ω6) in the PE (34%) and PC fractions (15%) in term neonates (≥37 weeks, n= 9). Compared to term neonates, preterm (<37 weeks, n=10) and DNI (≥37 weeks, n= 9) CBMC levels of both omega-3 and omega-6 LCPUFAs were significantly lower. In addition term neonates had significantly higher numbers of CD4 and CD8 leukocytes than preterm and DNI neonates, including naïve and memory lymphocytes. These deficiencies in vital LCPUFAs and immune subset numbers, may contribute to the immature status of the preterm and DNI immune system, and ultimately to compromised immune function. A preliminary study was conducted into the function of immune cells *in vitro* in media supplemented with omega 3 and omega 6 LCPUFAs. The results, while not conclusive, suggested that LCPUFAs were beneficial to lymphocytes in culture.

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Abbreviations

AA	-	Arachidonic acid
AGA	-	Appropriate for gestational age
ALA	-	Alpha linolenic acid
ARV	-	Antiretroviral (therapy)
ATL	-	Aspirin triggered lipoxin
AZT	-	Azidothymidine (zidovudine)
BHT	-	Butylated hydroxyl toluene
BPD	-	Bronchopulmonary dysplasia
CBMC	-	Cord blood mononuclear cells
CDC	-	Centre for Disease Control
COX	-	Cyclooxygenase
DHA	-	Docosahexaenoic acid
DHGLA	-	Dihomogamma linoleic acid
DNI	-	Definitely not infected
DPA	-	Docosapentaenoic acid
DTA	-	Docosatetraenoic acid
EPA	-	Eicosapentaenoic acid
FCS	-	Fetal calf serum
GLA	-	Gamma linolenic acid
^{3}H-Thy	-	Tritiated thymidine
HIV	-	Human Immunodeficiency Virus
IL-2	-	Interleukin 2
IVH	-	Intraventricular haemorrhage
LA	-	Linoleic acid
LCPUFA	-	Long chain polyunsaturated fatty acid

LGA	-	Large for gestational age
LOX	-	Lipoxygenase
LXA_4/B_4	-	Lipoxin A_4 and B_4
MTS	-	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
NEC	-	Necrotizing enterocolitis
OD	-	Optical density
p	-	Significance value
PES	-	Phenazine ethosulfate
PGD/E/H/J	-	Prostaglandins (D, E, H or J)
PHA	-	Phytohaemagglutinin
PLA_2	-	Phospholipase A_2
PMN	-	Polymorphonuclear
P/S	-	Penicillin and Streptomycin
r	-	Correlation coefficient
RDS	-	Respiratory distress syndrome
RPMI 1640	-	Roswell Park Memorial Institute
SGA	-	Small for gestational age
TCR	-	T-cell receptor

Chapter 1

Literature Review

1.0 Introduction

The importance of essential fatty acids in the diet and the clinical effect of a deficiency of these fatty acids on animal physiology were discovered in the late 1920s and early 1930s by George and Mildred Burr (Burr and Burr, 1929 and 1930). They found that rats fed on diets deficient in linoleic acid (LA-18:2ω6) had skin, feet and tail scaliness, kidney malfunction, growth retardation and impaired reproduction. Essential fatty acids refer to linoleic acid (found in the seeds of cereals and grains) and α-linolenic acid (found in flaxseed oil, green leafy vegetables and certain marine algae). The long chain fatty acids, LA and α-linolenic (ALA-18:3ω3) acids are the parent compounds of the long chain polyunsaturated fatty acids (LCPUFAs) arachidonic (AA) and docosahexaenoic acids (DHA) respectively. In turn AA and DHA are released from the cell membrane to form biologically active eicosanoids, lipoxins, neuroprotectins and resolvins.

In addition to playing an important structural role in the inner and outer leaflets of the cell membrane, LCPUFAs such as AA (20:4ω-6) and DHA (22:6ω-3) are involved in cell signalling mechanisms and ultimately cell function. They are therefore integral components of the membranes of many cell types including those of the neural, visual, vascular and the neonatal immune system (Diau *et al*, 2005; Bazan *et al*., 1990, Hirafuji *et al*., 2002 and Chung *et al*., 2005). The modulation of inflammation and pro-resolution of inflammation by ω6 and ω3 LCPUFAs has been well documented and reviewed as a complex integration of biochemical processes (Mukherjee *et al*., 2004; Marcheselli *et al*. 2003 and Serhan, 2005) and can no longer be simply categorised as "good" and

"bad" fatty acids or indeed as definitive inflammatory or anti-inflammatory agents. The AA-derived eicosanoid, leukotriene B_4 has been shown to induce differentiation and proliferation of human cord blood $CD34^+$ hematopoietic stem cells *ex vivo*, as well as increase the survival of these cells via an anti-apoptotic mechanism (Chung *et al.*, 2005).

It is therefore of importance to understand how a deficiency in LCPUFAs may influence the maturation of the fetal immune system, particularly in high risk pregnancies where the placental provision of LCPUFAs may be less than suitable for fetal development. Such pregnancies include preterm delivery (<37 weeks gestation) and pregnancies in which the foetus is placed at risk by maternal viral infection such as HIV.

Neonates of low gestational age are born at a time when their immune systems are not fully mature and are therefore not as immunocompetent as term neonates. Preterm neonates had lower numbers of CD3+ T-lymphocytes, particularly CD4+ T-helper cells than healthy neonates born at term (Wilson *et al.*, 1985 and Juretić *et al.*, 2001). This may account for the susceptibility of preterm neonates to non-specific and hospital-acquired infections post-natally (Juretić *et al.*, 2001). However, very preterm neonates (<32 weeks gestation) or very low birth weight neonates are also susceptible to inflammation resulting from post-natal mechanical injury during resuscitation or chronic mechanical ventilation as a result of very small and poorly formed lungs (Jobe and Ikegami, 1998). In response to injury, the subsequent release of proinflammatory mediators (cytokines) may have a negative influence on lung development resulting in bronchopulmonary dysplasia (Jobe and Ikegami, 1998).

Women who are HIV-positive are also at risk of delivering preterm neonates (Lorenzi *et al.*, 1998 and Temmerman *et al.*, 1994), which poses a greater risk of intrapartum exposure of the virus to the fetus (Kuhn *et al.*, 1997). However, it has become clear that even uninfected neonates born to HIV-positive women are not as immunocompetent as healthy neonates born to uninfected women. The uninfected neonates of infected mothers have been reported to have lower numbers of T-lymphocytes due the possible impairment in function of progenitor cells (Nielson *et al.*, 2001). In addition T-lymphocyte function was altered (Rich *et al.*, 1997 and Clerici *et al.*, 2000), susceptibility of T-cells to apoptosis was higher than in healthy neonates (Economides *et al.*, 1998) and the frequency of HIV-specific T-cells was augmented (Nielson *et al.*, 2001).

1.0.1 Thesis Aim

Although there is a well-documented pool of literature on the phenotypic and functional differences between term and preterm neonates, to date there is insufficient information on the impact of the loss of vital LCPUFAs on fetal immune system maturation. Furthermore the preterm neonatal mononuclear cell membrane fatty acid status at birth has never before been studied.

The main aims were as follows:

- To establish the fatty acid composition of cord blood mononuclear cells between healthy term, preterm neonates and uninfected term neonates born to HIV positive women.
- To determine the cord blood immunophenotype and subset numbers with regards to pregnancy status and birth outcome.

The LCPUFA composition of the inner and outer leaflets of the mononuclear cell membranes were analysed by gas chromatography. The studies yielded novel data on the inner and outer leaflets of cord blood mononuclear cell membranes.

The quantification of the immunological profiles of cord blood was conducted by flow cytometry. In addition, when appropriate amounts of mononuclear cells became available, the *in vitro* cell viability and functional response of the cells were assessed with the use of a non-radioactive cell viability colorimetric assay and flow cytometry.

Pregnant women in labour were recruited in the delivery suite at Newham University Hospital NHS Trust, London. Signed informed consent was given prior to cord blood being collected. This project was authorised by the East London Health Ethics Committee.

1.1 Background Literature

1.1.1 Fatty acids

Fatty acids are lipid nutrients. They are principally found in the cell membrane bilayer of all cells, and are of great importance in the structure, function and maintenance of the cellular environment. Essential fatty acids cannot be produced by the body, and thus have to be procured from the diet (Groff *et al.*, 1995). Linoleic (LA; 18:2ω6) and α-linolenic (ALA; 18:3ω3) acids are referred to as essential fatty acids.

1.1.1.1 Fatty acid chain lengths and degree of saturation

All fatty acids comprise of a hydrocarbon chain with a range of chain lengths from 8 to 24 carbon atoms. Each hydrocarbon chain begins with a methyl group and terminates in

a carboxylic acid end group, and this confers polarity to the molecule (Groff *et al.*, 1995). Fatty acids exist in three forms:

a) Saturated fatty acids (SFA) refer to hydrocarbon chains devoid of double bonds;

b) Monounsaturated fatty acids (MUFAs) have one double bond in the chain, and;

c) Polyunsaturated fatty acids (PUFAs) have two or more double bonds.

Different families of PUFAs are distinguished by the positions of the double bond furthest from the carboxyl group, or those closest to the methyl group (Grammatikos *et al.*, 1994). They are commonly named according to the position of this double bond by counting from the methyl terminal, with the methyl carbon (n or omega (ω) carbon) as 1. Most naturally occurring fatty acids exist in the cis form (U-like orientation), whilst the trans form (linear) exist in some partially hydrogenated fats and oils (Groff *et al.*, 1995). These geometrical isomerisms are now more commonly referred to as the Z and E configurations respectively (Leighfield, 1992).

Double bonds are located in different positions on the hydrocarbon chain. The shorthand notation for fatty acids therefore, includes two numbers separated by a colon. The number before the colon refers to the number of carbon atoms in the chain, whilst the number after the colon refers to the number of double bonds.

Therefore: **20:4 ω6**

indicates that there are 20 carbon atoms in the chain, 4 double bonds, and the position of the first double bond from the methyl end is on carbon 6 and is denoted by ω6 or in some cases n-6. Further examples of LCPUFAs of the omega 6 and omega 3 families are represented in figure 1.1 below.

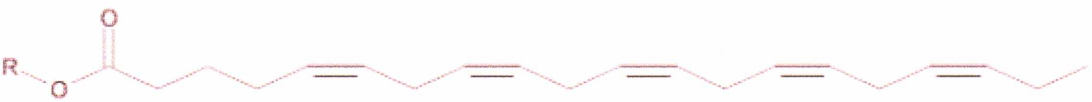
9,12-Octadecadienoic acid (linoleic acid) - ω6

8,11,14-Eicosatrienoic acid (dihomo gamma-linolenic acid) - ω6

5,8,11,14-Eicosatetraenoic acid (arachidonic acid) - ω6

9,12,15-Octadecatrienoic acid (α-linolenic acid) - ω3

4,7,10,13,16,19-Docosahexaenoic acid (cervonic acid) - ω3



5,8,11,14,17-Eicosapentaenoic acid - ω3

Figure 1.1: Two-dimensional examples of long chain polyunsaturated fatty acids. Where the omega 6 fatty acids are distinguished by the first double bond on the sixth carbon atom from the methyl terminal end, and omega 3 LCPUFAs are determined by the first double bond at carbon 3 from the methyl terminal.

1.1.1.2 Cell membrane lipids

Fatty acids exist as free fatty acids as well as components of:

1) Neutral lipids, which are comprised of the mono, di and triacylglycerols (triglycerides) as well as free fatty acids, wax esters and sterols;
2) Phosphoglycerides, which consist of the choline, ethanolamine, inositol and serine phosphoglycerides, and
3) Sphingomyelins and cerebrosides.

Triacylglycerol molecules ($CH_2COOCR1CHCOOCR2CH_2COOCR3$) consist of three fatty acids (R1, R2 and R3), which tend to be esterified to particular positions on a glycerol backbone; for example, PUFAs tend to accumulate at position 3 in most mammals (Leighfield, 1992).

Phosphoglycerides ($CH_2COOR1CHCOOR2CH_2PO_4R3$) belong to a class of phosphorous containing lipids (phospholipids) and in mammals are mainly involved in membrane structures together with unesterified (free) cholesterol (Leighfield, 1992). The R1 and R2 positions contain fatty acids, while the R3 group is part of the hydrophilic head groups, which contain some charge. The R3 group is also responsible for the naming of the phosphoglycerides, which are all synthesised from phosphatidic acid (Mathews and van Holde, 1990). Examples of these are:

$R3 = CH_2CH_2NH_3^+$ (phosphatidylethanolamine (PE))

$R3 = CH_2CH_2N(CH_3)_3^+$ (phosphatidylcholine (PC))

Phospholipids are important components of lipid membranes because of their amphiphilic properties, which confers selective permeability to the cell membrane (Mathews and van Holde, 1990).

Sphingomyelin and cerebrosides are also common phospholipids that are found in nervous tissue and they are built on long-chain amino alcohol sphingosine rather than glycerol backbones (Mathews and van Holde, 1990). Cardiolipin is a phosphoglycerides that is found in the inner and outer membranes of mitochondria[2] and preferentially accumulates DHA.

PC and PE represent the majority of the membrane phospholipids and are followed by smaller quantities of phosphatidylserine (PS) and phosphatidylinositol (PI), the latter important in signal transduction[2]. In the Human erythrocyte membrane, the cytosolic leaflet (inner) contains mainly PE and PS, while the exoplasmic (outer) leaflet contains PC and sphingomyelin as well as cholesterol, which are fused ring structures that contribute to rigidity[2] (Mathews and van Holde).

1.1.2 Dietary sources of fatty acids

It has been suggested that human beings evolved on a diet with a 1:1 ratio of ω6 to ω3 fatty acids, whereas modern Western diets are 15 to 16 times higher in ω6 fatty acids (Simopoulos, 2002).

Raw materials from which food fats are derived are mainly storage fats of land and marine mammals, fish and seed oils (Leighfield, 1992). These are then made suitable by extraction and purification. Some naturally occurring fatty acids and their dietary sources are shown in table 1.1.

Table 1.1: Some naturally occurring fatty acids (adapted from Groff *et al.*, 1995).

Shorthand notation	Fatty acid	Formula	Dietary Source
16:1 ω-7	Palmitoleic acid	CH_3-$(CH_2)_5$-CH=CH-$(CH_2)_7$-COOH	Marine animal oils, plant and animal fats.
18:1 ω-9	Oleic acid	CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-COOH	Plant and animal fats.
18:2 ω6	Linoleic acid	CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH	Corn, safflower, soybean, cottonseed, sunflower seed and peanut oil.
20:4 ω6	Arachidonic acid	CH_3-$(CH_2)_3$-$(CH_2$-CH=CH$)_4$-$(CH_2)_3$-COOH	Small amounts in animal fats.
18:3 ω3	α-Linolenic acid	CH_3-$(CH_2$-CH=CH$)_3$-$(CH_2)_7$-COOH	Linseed, soybean, green plants and other seed oils.
20:5 ω3	Eicosapentaenoic acid	CH_3-$(CH_2$-CH=CH$)_5$-$(CH_2)_3$-COOH	Marine algae and fish oils.
22:6 ω3	Docosahexaenoic acid	CH_3-$(CH_2$-CH=CH$)_6$-$(CH_2)_2$-COOH	Animal fats as phospholipid component and fish oils.

Fish can either store their reserve fats as triacylgycerols in the liver (e.g. lean fish like cod) or in the flesh such as oily fish like mackerel and herring. Fish, shellfish and marine plants have abundant quantities of fatty acids which are metabolites of ALA and differ from mammalian and avian fats by having a larger content of LCPUFAs of the ω3 family such as eicosapentaenoic acid (EPA) and DHA (Hepburn *et al.*, 1986, Ackman, 1989 and Drevon, 1992).

The structural lipids, present in the cell membranes of animal and plant tissues are mainly phospholipids and glycolipids, and they have a much higher content of PUFA than the storage lipids (Leighfield, 1992). Due to their role in membranes, the fatty acid composition of lean meat is less variable and less susceptible to dietary influence than storage fats (Leighfield, 1992). Ruminant meat for example is a source of arachidonic acid (AA) whilst the adipose tissue of pigs, poultry and ruminants contain little AA but large amounts of saturated and monounsaturated fatty acids. The fatty acid content of sheep, cow and goat's milk have large amounts of short and medium chain fatty acids and small quantities of PUFAs (Leighfield, 1992).

The fatty acid composition of seed oils vary, usually with one type of fatty acid predominating, whilst the lipid structure of plant leaf membranes are comprised mainly of palmitic acid (16:0), palmitoleic acid, LA and ALA (Groff *et al.*, 1995). Of these, the most important fatty acids for human consumption are, LA and ALA, as they are the precursors to the long chain polyunsaturated ω6 and ω3 fatty acids, respectively (Innis, 1991). LA is sourced mainly from cereals and grains, while ALA is found in green food and certain marine algae. Human milk is another natural source of ω3 and ω6 LCPUFAs (Crawford *et al.*, 1976), however the levels are modulated by maternal diet (Finley *et al.*, 1985 and Francois *et al.*, 1998).

1.1.3 Digestion of dietary fats

1.1.3.1 Hydrolysis of fats in the stomach and small intestine

Dietary lipids contain approximately 97% triglycerides (Thomson *et al.*, 1993) as well as phospholipids (mainly phosphatidylcholine) and sterols (Groff *et al.*, 1995). The digestive process of lipids begins in the stomach and is completed in the lumen of the small intestine (Groff *et al.*, 1995). The digestive enzymes at these sites are lingual lipase and pancreatic lipase respectively; although it has also been shown that pancreatic colipase and carboxyl ester lipase rapidly converts EPA and AA into free fatty acids and monoacylglycerols (Chen *et al.*, 1990). Lingual lipase hydrolyzes triglycerides to free fatty acids and partial glycerides and is an especially important enzyme in the milk fat digestion by neonates (Fink *et al.*, 1984) and sucking seems to enhance enzyme production (Smith *et al.*, 1986). The products of the partial digestion of lipids combine with bile salts forming negatively charged polymolecular aggregates called micelles (Groff *et al.*, 1995).

1.1.3.2 Absorption and transport of hydrolysed lipids

The absorption and transport of lipids have been extensively reviewed (Leighfield, 1992; Groff *et al.* 1995 and Thomson *et al.* 1993) and is depicted in figure 1.2 as a summary of these reviews. The micelles formed during hydrolysis are sufficiently water soluble to pass through the unstirred water layer bathing the absorptive cells (intestinal mucosal cells or enterocytes) of the small intestine (figure 1.2). The lipid contents of the micelles diffuse out of the micelles and into the enterocytes along a concentration gradient (Groff *et al.*, 1995). Triglyceride-rich osmiophilic particles of about 150 to 5000 Å in diameter which become visible within the endoplasmic reticulum and the golgi apparatus, exit to the lateral intercellular spaces and move through the gaps in the

1.1.4 Metabolism of LCPUFAs

The fatty acid composition of developing tissues is dependent on the quantity and type of lipid in the diet, the activity of the desaturase enzymes responsible for the synthesis of LCPUFAs from 18-carbon fatty acids as well as the partitioning of the ω3 and ω6 fatty acids among oxidation, desaturation and acylation (Innis, 1991).

1.1.4.1 The metabolic pathways for the synthesis of AA and DHA

Plants unlike animals are able to insert double bonds into oleic acid (18:1ω9). A Δ^{12} desaturase converts oleic acid to LA (18:2ω6) while a Δ^{15} desaturase converts linoleic acid into ALA (18:3ω3) (Miles and Calder, 1998). Animal tissues are unable to carry out this process and therefore have to procure LA and ALA from the diet. LA and ALA are essential due to the fact that vertebrates lack the Δ^{12} and Δ^{15} desaturases, which incorporates double bonds at these molecular positions i.e. carbon 12 and carbon 15 (Groff *et al.*, 1995). These enzymes are found only in plants and this makes desaturation in animal cells beyond Δ^{9} impossible unless the desaturases are taken from the diet. Furthermore fatty acid chains can also be elongated with the enzymatic addition of two carbons at the carboxylic end of the fatty acid chain (Groff *et al.*, 1995). Most fatty acids have even numbered carbon chains. Odd numbered carbon chains of fatty acids also occurs naturally in some sources such as certain fish (menhaden, mullet and tuna) as well as the bacterium *Euglena gracilis* (Groff *et al.*, 1995)

The precursors of AA and DHA are LA and ALA respectively (Figure 1.2). However, these synthesis pathways are rate limited by the Δ6 desaturase (Marcel *et al.*, 1968 and Hassam *et al.*, 1977). Figure 1.3, shows that Δ6 desaturase is necessary in the final desaturation step in the production of DHA, in which 24:6ω3 is produced and

1.1.4.2 Markers for LCPUFA insufficiencies

In instances when the intake of LA and ALA are insufficient, oleic acid (which uses the same desaturases) is converted to Mead acid (20:3ω9) (Mead and Slaton, 1956). Mead acid or the Mead acid/AA ratio is used as a biochemical marker of EFA deficiency (Holman, 1977). On the other hand, diets that are specifically deficient in ω3 fatty acids or rich in LA, are biochemically marked by decreased DHA and increases in docosatetraenoic acid (22:4ω6) and docosapentaenoic acid (22:5ω6); and DHA deficiency is indicated by the ratios of 22:5ω6/DHA and 22:5ω6/22:4ω6 (Fiennes *et al.*, 1973 and Bourre *et al.*, 1992). In addition to the low yield of AA and DHA from the rate limited desaturation and elongation pathway (Sinclair 1975), disruption of the desaturation mechanism has been shown to be pathological in nature and was found in diabetes (Holman *et al.*, 1983 and Gordon *et al.*, 1995), Crohn's disease (Clemmesen *et al.*, 2000), cirrhosis (Geerling *et al.*, 1999), cystic fibrosis (Strandvik *et al.*, 2001) and Zellweger's syndrome and adrenoleukodystrophy (Braiterman *et al.*, 1999 and Martinez, 1992).

1.1.5 Cell membrane LCPUFAs and cell function

1.1.5.1 Cell membrane fluidity

Cell membrane fluidity is the measure of the freedom of movement of proteins and lipids within the membrane, and this may be altered by the fatty acid content of the membrane (Brasitus *et al.*, 1985).A primary consequence of fatty acid supplementation *in vitro* is the modification of the membrane fatty acid composition (Grammatikos *et al.*, 1994). LCPUFAs integrate into cell membranes and influence membrane fluidity, ion channel flow and cell surface receptor function (Zaloga, 1999). Workers have shown previously that structural and physical properties vary with chain length and degree of unsaturation, and thus fatty acid compositional changes can affect the structure and

function of the membrane (Grammatikos *et al.*, 1994). LCPUFAs with one or more double bonds do not fit tightly together, as a result of the cis bends in the chain which interferes with packing, whereas saturated chain fit parallel and therefore, tightly together (Becker *et al.*, 2005).

Workers have shown that while membrane fluidity increases during cell division, LCPUFAs such as dihomo-γ-linoleic acid (20:3ω6), AA, eicosapentaenoic acid (20:5ω3; EPA) and DHA, which should increase membrane fluidity are actually inhibitory to the growth of several cancer cell lines (Grammatikos *et al.*, 1994; Das, 1999).

1.1.5.2 Cell signalling

Protein and enzyme dependent cell functions are modulated by signal transduction, which is in turn dependent on the structural properties of the membrane. Therefore dietary ω-6 and ω-3 fatty acids influence cell membrane profiles and consequently the expression of genes (Simopoulous, 1996).

Cell signalling pathways probably evolved to respond rapidly to environmental changes. Zaloga (1999) hypothesises that that cellular receptors were activated by environmental ligands, such as fatty acids, which could have been used to modulate metabolic pathways and control cell division (Zaloga, 1999). Dietary lipids specifically long and medium-chain triglycerides have been shown to modulate cell signalling in neutrophils through calcium mobilisation and protein kinase C activation (Wanten *et al.*, 2001). DHA has been postulated to be involved in phospholipid mediated cell signalling due to it preferential incorporation into the synaptic membrane phospholipids of rat brains in response to Cholinergic activation when compared to saturated palmitic acid (Jones *et*

al., 1997). Ballou et al. (1996) and others have also reviewed the function of ceramide in signal transduction and it role in inflammation. More recently, information on lipid rafts in cell membranes as microdomains in signal transduction have been demonstrated. Lipid rafts are portions of the cell membrane that contain sphingolipids and cholesterol into which proteins are anchored. Lipid rafts have been shown to be involved in the CD3 activation of diacylglycerol and cytosolic calcium production, major events in T-cell activation (Rouquette-Jazdanian *et al.*, 2002).

1.1.5.3 Gene expression

The effects of nutrition can be exerted at many stages between transcription of the genetic sequence and production of a functional protein (Hesketh *et al.*, 1998). These may manifest as either altered mRNA stability, altered translation of the mRNA or changes in proteolytic breakdown of the protein (Hesketh *et al.*, 1998).

Polyunsaturated fatty acids have a direct influence on the molecular events governing the expression of genes involved in fuel partitioning, during which metabolic fuels are directed away from storage and towards oxidation (Clarke, 2000). Studies have revealed that *in vivo* high fat diets rich in ω-6 and particularly ω-3 fatty acids induced increases in peroxisomal acyl-CoA oxidase in rat liver and other tissues (fatty acid oxidation); whilst in mice fed a corn oil diet (high in 18:2ω6; LA) the expression of hepatic fatty acid synthase gene was significantly reduced compared to mice given a high glucose diet without fat ($p<0.05$) (Clarke *et al.*, 1997). Some examples of lipogenic genes that are regulated by PUFAs are shown in table 1.2. *In vitro* studies with cultured primary hepatocytes showed that 18:3ω3, 18:3ω6, 20:4ω6 and 20:5ω3 effectively suppressed the fatty acid synthase gene (Jump *et al.*, 1994).

Table 1.2 Examples of genes and proteins that are regulated by long-chain fatty acids (adapted from a review by Pègorier, 1998)

	Up-regulated	Down-regulated
Liver Fatty acid transport	Fatty acid binding protein Apoprotein A II Lipoprotein lipase	Apoprotein A I Apoprotein C III
Mitochondrial oxidation and ketogenesis	Acyl-CoA synthase Carnitine palmitoyltransferase I Medium-chain acyl-CoA dehydrogenase Mitochondrial hydroxymethyl-glutaryl-CoA synthase	
Peroxisomal β-oxidation	Acyl-CoA oxidase Enoyl-CoA hydratase 3-Ketoacyl-CoA thiolase	
Lipid Synthesis	Stearoyl-CoA desaturase	Fatty acid synthase Acetyl-CoA carboxylase ATP-citrate lyase Spot 14 Pyruvate kinase[1]
Other	Adipocyte lipid-binding protein (aP2) Keratinocyte lipid-binding protein (MAL-1) Phosphoenolpyruvate carboxykinase (PEPCK) Glucose transporter GLUT 4 Uncoupling protein-2	Transferin

[1]- Clarke (2000)

PUFA regulation of gene transcription is a ligand-mediated event. Fatty acids were found to activate a steroid receptor called the peroxisome proliferator activated receptor (PPAR) (Göttlicher *et al.*, 1992) which as first isolated from mice (Isseman and Green, 1990). When PPAR is bound to a ligand (figure 1.4), it then forms the PPAR-RXR heterodimer (RXR is the retinoid X receptor); which associates with a DR-1 concensus sequence known as peroxisome proliferator response elements (PPRE) located within the nucleus (Moya-Camarena and Belury, 1999) initiating gene expression. The ligands that bind PPAR involve a wide array of factors including fatty acids, fibrates,

prostaglandins, leukotrienes and oxidized fatty acids (Keller *et al.*, 1993; Kliewer, *et al.*, 1997 Clarke, 2000). Liver-type fatty acid binding protein (L-FABP) is said to be involved in the transport of fatty acids and peroxisome proliferators from the cytosol into the nucleus for interactions with PPARs (Wolfrum *et al.*, 2000).

Three subtypes of PPAR (α, β [or δ in mice], and γ) are found in amphibians, rodents fish and humans (Moya-Camarena and Belury, 1999). PPARα is mainly expressed in the liver, kidney and heart. The activation of PPARα in rats and mice is associated with enhanced hepatocarcinogenesis, although these adverse conditions are not noted in humans (Moya-Camarena and Belury, 1999). This indicates possible species differences in the regulation of gene expression by PUFAs.

Although it was initially thought that PPARs regulated the fuel portioning genes, it is now known that this is due to the changes in expression and nuclear localisation of the transcription factor, sterol-regulatory element-binding protein-1 (SREBP-1) (Worgall *et al.*, 1998 and Xu *et al.*, 1999). PPARs are involved in the regulation of genes involved in tumor suppression as well as inflammatory reactions. The prostaglandin D_2 metabolite, 15-deoxy$^{\Delta 12,14}$ prostaglandin J_2 (15d-PGJ_2) is a PPARγ activator that was shown to inhibit the inflammatory CXC chemokine mRNA expression in endothelial cells in vitro (Marx *et al.*, 2000). The binding of 15d-PGJ2 also resulted in the inhibition of mitogen-induced IL-2 expression in T-cells in a dose dependent manner, most likely by allowing PPAR to compete with DNA in the binding of the T-cell specific transcriptional factor NFAT thereby blocking transcription (Yang *et al.*, 2000).

lymphocyte population, CD8+population as well as the increase in Th2 (IL-5 and IL-10) production and the increases in Th1 (IL-2 and interferon-γ (IFN-γ)) production. Feeding the mice with 40% restricted corn oil and 5% fish oil partially accomplished these effects. The data indicates that a 40% resistricted diet of fish oil may delay the onset of autoimmune kidney disease by suppressing both the Th1 and Th2 cytokine production (Jolly and Fernandes, 1999).

The effects of DHA ethyl esters on T-lymphocytes at contact hypersensitivity sites were examined using anti-CD4 antibodies on the ears of mice by Tomobe *et al.*, 2000. DHA but not EPA was found to reduce the swelling. DHA also reduced the inflitration of CD4+ T-lymphocytes into the ears as well as reducing the expression of inflammatory cytokines IFN-γ, IL-6, IL-1β and IL-2 mRNA (Tomobe *et al.*, 2000).

Sasaki *et al.* (1999) found that feeding weanling mice on a 10% fat diet (varying amounts of DHA and LA) resulted in a decreased surface expression of CD4 and CD8 on splenic T-cells with an increase in DHA concentration. CD28 expression increased (Sasaki *et al.*, 1999). CD28 is a T-cell co-stimulator molecule which is expressed on T-cell subsets (Benjamini *et al.*, 2000).

LCPUFAs are also important regulators of inflammation. Inflammation is a fundamental protective process that is essential in health and disease. Inflammation usually presents clinically as oedema, hyperaemia and infiltration by polymorphonuclear (PMN) leukocytes e.g. neutrophils. Over the decades eicosanoids were thought to be the main regulatory components of inflammation. However more recently, local cellular and molecular events that govern the formation and action of

local mediators that can serve as local endogenous mediators of resolution have been discovered (Serhan, 2005b). These compounds can control the duration and magnitude of inflammation.

1.1.6.1 Eicosanoids

The cells of the immune system are present as circulating cells in the blood and lymph, as anatomically defined collections in lymphoid organs (thymus, spleen and lymph nodes) or as scattered cells in virtually all tissues (Miles and Calder, 1998). The principle cells of the immune system are T- and B-lymphocytes, natural killer (NK) cells, dendritic cells, mononuclear phagocytes (monocytes and macrophages) and granulocytes (neutrophils, eosinophils and basophils) (Miles and Calder, 1998).

Nutritional interest in ω-3 PUFAs have increased due to their hypolipidaemic and antithrombotic effects (Groff *et al.*, 1995). An ω-3 PUFA of particular interest is EPA, because it is a precursor of the physiologically important eicosanoids. Fish oils are particularly rich in these fatty acids (Groff *et al.*, 1995).

Eicosanoids are fatty acids that are comprised of 20 carbon atoms, and they include the physiologically important families of substances called prostaglandins, thromboxanes and leukotrienes (Groff *et al.*, 1995). These are formed from precursor fatty acids, by the incorporation of oxygen atoms within the fatty acid chains (oxygenation reactions). The enzymes catalysing these reactions are oxygenases (Groff *et al.*, 1995). The most important fatty acid serving as a precursor for eicosanoid synthesis is AA. It is oxygenated via two pathways: (a) the cyclooxyenase (COX) pathway (which results in the formation of prostaglandins and thromboxanes) and (b) the lipoxygenase (LOX) pathway resulting in leukotriene formation (Groff *et al.*, 1995). Prostaglandins as well

as thromboxanes exhibit a wide range of physiologic actions, including the lowering of blood pressure, diuresis, blood platelet aggregation, effects on the immune and nervous system and gastric secretions, and the stimulation of smooth muscle contractions (Groff *et al.*, 1995).

Leukotrienes are involved in the contraction of the respiratory, vascular and intestinal smooth muscle. Therefore the amount of free unesterified arachidonate available is necessary for eicosanoid formation (Groff *et al.*, 1995). Cellular concentrations of free fatty acids are often not adequate and have to be released from membrane glycerophosphatides, by a specific hydrolytic enzyme called phospholipase A_2. The most important phosphoglycerides acting as sources of AA in cells, are phosphatidylcholine and phosphatidylinositol (Groff *et al.*, 1995).

The role of LCPUFAs, particularly AA and DHA has been reviewed many times over the years (Calder, 2005; Calder, 2003; Calder *et al.*, 2002; Grimm *et al.*, 2002 and Miles and Calder, 1998). Until very recently the roles of ω-6 and ω-3 LCPUFAs were compartmentalized into pro-inflammatory and anti-inflammatory precursors respectively.

Eicosanoids are known to be important mediators of immune function and regulators of platelet aggregation, blood clotting, leukocyte chemotaxis and inflammatory cytokine production (Calder, 2003). Prostaglandins and leukotrienes derived from AA had previously been shown to be predominantly pro-inflammatory (Rola-Pleszczynski and Stankova, 1992; Rola-Pleszczynski and Lemaire, 1985) and then as understanding of prostaglandin physiology increased, they were seen as part of a complex regulatory network (Tilley *et al.*, 2001) with both pro- and anti-inflammatory properties,

depending on the timing of the production of different eicosanoids as well as on the sensitivity of the target cells to these eicosanoid concentrations (Calder *et al.*, 2002).

Inflammation has been linked to cardiovascular disease, Alzheimer's disease and cancer (Israelian-Konaraki and Reaven, 2005, Irizarry, 2004 and Smith and Missailidis, 2004). Coronary heart disease, major depression, aging and cancer are characterized by an increased level of interleukin 1 (IL-1), a proinflammatory cytokine; while autoimmune diseases like Crohn's disease, ulcerative colitis and lupus erythematosis are characterized by high levels of IL-1 and the proinflammatory leukotriene LTB_4 produced by ω6 fatty acids (Simopoulos, 2002).

Prostaglandins regulate cellular growth, differentiation and homeostasis (Kaur *et al.*, 1999). Upon inflammatory stimulation, AA is released from the cell membrane by phospholipase A_2 (figure 1.4) and converted to PGH_2 by the cyclooxygenase enzymes (COX-1 and COX-2) (Harris *et al.*, 2002) together with specific isomerases (Tilley *et al.*, 2001). PGH_2 is then converted by cell specific prostaglandin synthases into various prostaglandins (PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$) (Kaur *et al.*, 1999) and thromboxane A2 (Calder, 2005) and receptors all of which work in sites close to their synthesis. The 2-series prostaglandins are so named because AA loses 2 unsaturated bonds during the cyclooxygenase reaction (Flower and Perretti, 2005). AA can also be converted to leukotrienes via the 5-lipoxygenase (5-LOX) pathway, as well as to lipoxin A_4 (discussed later) via the 15-LOX pathway (figure 1.4).

PGE_2 is one of the most studied prostaglandins. Kuratko and Becker (1998) showed that feeding rats diets rich in corn oil not only increased the colonic lymphocyte membrane concentration of LA and AA, but also increased the levels of PGE2 production.

Through experiments using knock-out mice for COX-1 and COX-2 and prostaglandin receptors, PGE_2 has been shown to be a potent modulator of immune function (Calder, 2003 and Harris *et al.*, 2002). He and Stuart (1999) found that PGE_2 sensitive CD4+ cells produce low amounts of interleukins-2 and -4 (IL-2 and IL-4), while PGE_2 resistant CD4+ cells produce high amounts of these cytokines. CD45RA expression was lower in the PGE_2 sensitive cells than in the resistant ones (He and Stuart, 1999). Furthermore, PGE_2 resistant cells became PGE_2 sensitive when IL-2 and IL-4 were removed using specific antibodies (He and Stuart, 1999).

PGE_2 has been shown to inhibit the Th1 response, which is an inflammatory or cell mediated response of T-cells, in favour of the Th2 response (humoral) (Calder, 2003 and Kaur *et al.*, 1999). Inhibition of the Th1 response involves inhibition of the production of inflammatory cytokines such as interleukin 2 (IL-2) and IFNγ, while enhancing the Th2 response by increasing the production of IL-4, IL-5 and IL-10 (Calder, 2003). This is regarded as a simplistic explanation as the regulation of the inflammatory systems is a complex and intricate mesh of stimuli, signals, presence of other mediators and specific prostaglandins. Paradoxical findings for the contribution of AA and other n-6 LCPUFAs in inflammation and immunosuppression are common (Harbige, 2003; Kuratko and Becker, 1998).

The conversion of AA to leukotriene A_4 (LTA_4, in figure 1.5) by the 5-LOX enzyme leads to the synthesis of LTB_4 by LTA_4 hydrolase or the conjugation of LTA_4 with glutathione by LTC_4 synthase to form LTC_4 and further to LTD_4 and LTE_4 (Goulet *et al.*, 1994). In the early nineties, isolation of leukotrienes from the sites of inflammation

neutrophils at the site of inflammation. Therefore leukotrienes are intrinsic inflammatory components.

1.1.6.2) Anti-inflammatory mediators of resolution

For several decades lipids were thought of to be the "bad guys" of inflammation and drugs such as glucocorticoids and non-steroidal anti-inflammatory drugs were thought to work clinically by blocking them (Serhan, 2005b). However, work has since been done to show that LCPUFAs such as AA are just as involved in anti-inflammatory processes as they are implicated in pro-inflammatory senarios.

When an injury occurs, polymorphonuclear cells (PMN) such as neutrophils, enter the site of injury as a host defence mechanism and this is called inflammation. Acute inflammation in healthy subjects is a normal self-limiting process and it is only in aberrant or pathologic conditions that there is an excess of PMN cells entering the site of injury, resulting in chronic inflammation (Serhan, 2005b).

a) Lipoxins

Lipoxins are trihydroxytetraene-containing mediators generated from AA during cell-to-cell interactions (figure 1.6) via transcellular biosynthesis (Serhan, 2005b). Lipoxins are ultimately generated via products of the COX-2 pathway.COX-2 derivatives produced increases in lipoxin A_4 (LXA_4) and aspirin-triggered lipoxin (15-Epi-lipoxin; ATL) in murine models (Fukunaga *et al.*, 2005).

Levy et al. (2001) showed that in PMNs exposed to PGE2 in clinical and experimental exudates, there was a temporal dissociation when PG and leukotrienes (from 5-LOX biosynthesis) anticeded and eventually switched to the 15-LOX production of LXA_4 and

ATL, ATL-15-epi-pheoxy analog, lipoxin B_4 (LXB4) and LXB_4-methoxy analog, was shown to block TNF-α secretion by Human T-cells (Ariel *et al.*, 2003). TNF-α, is a cytokine that is has antitumour activity as its name suggests, but it is also involved in inflammation, infection, immune modulation and even septic shock (Aggarwal and Reddy, 1994). LXA4, LXB4, ATL and their stable analogs also mediate pro-resolution processes by stimulating the uptake of apoptotic PMN cells by macrophages (Godson *et al.*, 2000 and Mitchell *et al.*, 2002). Therefore LX and ATL are also important initiators f the clean-up mechanism in the resolution of inflammation.

Thus, inhibitors that block inflammation, such as COX-1 and COX-2 inhibitors and glucocorticoids are actually preventing the synthesis of resolving mediators as well as inflammatory mediator. In murine models, COX-2 expression was increased in the lungs in an acute lung injury scenario, and when COX-2 was pharmacologically or genetically inhibited, resolution of the acute lung injury was also impeded (Fukunaga *et al.*, 2005). Recently an American court ordered Merck and Co. to pay an excess of 250 million U.S Dollars to the widow of a man prescribed Vioxx[3]. Vioxx is a COX-2 enzyme inhibitor prescribed for arthritic pain, and was approved by the FDA in 1999 but in 2004 was shown to be highly associated with increased risks of heart failure and stroke[4].

In experiments using murine dorsal skin pouches (experimental contained inflammation), workers found that after 4 hours the PMN cells began to drop off within the exudates (Serhan, 2002). Marcheselli *et al.* (2003) conducted ground-breaking experiments using a mouse stroke model to identify the docosahexaenoate-oxygenation derivatives. The workers induced an hour of middle cerebral artery occlusion in mice, followed by reperfusion, when free DHA was actively released from brain membrane phospholipids. The reperfusion was followed by the production of novel docosanoids including docosatriene (figure 1.9) and in models treated with aspirin, resolvin was found (Marcheselli *et al.*, 2003). They found that 10,17S-docosatriene was a potent inhibitor of PMN infiltration into the site of injury as well as an inhibitor of pro-inflammatory gene activation in neural cells in culture (Marcheselli *et al.*, 2003). Docosatriene infusion reduced stroke volume by 50% after 48 hours and therefore elicited neuroprotection *in vivo* (Marcheselli *et al.*, 2003).

Mukherjee *et al.* (2004), found that in human ARPE-19 cells (retinal pigmented epithelium) 10,17S-docosatriene inhibited oxidative stress induced by apoptosis as well as cytokine-triggered pro-inflammatory COX-2 gene promoter induction. The addition of DHA to the medium also provided strong cytoprotection when the retinal pigmented epithelial cells were confronted with oxidative stress (Mukherjee *et al.*, 2004).

The end of the second week of the development of the embryo is characterised by the extension of the cytotrophoblast cells into syncytiotrophoblast and this is called the primary chorionic villi and is the first stage in the development of the chorionic villi of the placenta (Moore and Persaud, 1998). This is followed by the development of the secondary and tertiary chorionic villi covering the chorionic sac, and a complete vascular network is established by the end of the fourth week (Moore and Persaud, 1998). The uterus, chorionic sac and placenta enlarge as the fetus grows.

The normal evolution of the functional immune system begins with the development of the thymus which begins at approximately 8 weeks gestation, which is quickly followed by the production of haematopoietic cells and the development of lymphocytes occurs at 8.5 to 9.5 weeks gestation (McNamara, 1999). At birth and into childhood the thymus is a fairly large organ and during late childhood it begins to involute (Moore and Persaud, 1998) but remains responsible for the diversification and priming of T-cells (Harbige, 2003).

1.1.8 Placental transfer of LCPUFAs to the fetus

Scientific interest in the development and nutrition of the fetus dates back to one of the fathers of medical science Claudius Galen (circa 130-201 AD) a Greek Physician in Rome who wrote "On The Formation of the Foetus" and in it described structures that we now know as the allantois, amnion and placenta (Moore and Persaud, 1998).

Apart from water, carbohydrates and amino acids (figure 1.12) the placenta selectively transfers PUFAs into the foetus, *in utero* (Crawford *et al.*, 1976). The proportion of AA received on the fetal side of the placenta is more than doubled from mid to late gestation (figure 1.11 below) and importantly, the level of LA is halved; a selective process is

LCPUFAs are crucial as structural components of the cell membrane of immune cells as they contribute to both directly and indirectly to immune function, particularly as mediators of inflammation. They are therefore important nutrients in the growth and development of the fetus as well as fetal immune system maturation. This is made all the more clear by the preferential transfer of AA and DHA to the fetus by the placenta. This remarkable biomagnification of AA by the placenta may well serve the immune system development of the fetus, and evidence for this possibility will be presented further in this thesis. Studies have shown that there is preferential incorporation of AA and LA during lymphocyte growth and proliferation (Calder, 1994 and Rode *et al.*, 1982) although feeding with fish oil increases the uptake of omega 3 fatty acids into the lymphocyte membrane at the expense of AA (Endres *et al.*, 1989). These studies were conducted in animal models and on lymphocytes from healthy adult volunteers respectively. Furthermore in mouse thymus there is preferential accretion of AA during early growth and development (Harbige, 2003).

There is a positive correlation between maternal PUFA consumption and neonatal PUFA status and pregnancy is associated with a decrease in biochemical PUFA status and normalisation thereafter is slow (Hornstra, 2000). Therefore it is of importance to remember that in pregnancies in which LCPUFA status in the mother may already be compromised, for example due to high turnover rates, the fetus would no doubt suffer deficits in the supply of LCPUFAs.

1.1.9 High risk pregnancies and the resultant fetal and neonatal immune system

A "high risk" pregnancy is one in which the mother or fetus has a disorder which puts one or both at an increased risk of morbidity or mortality (Johnstone, 1992). Preterm (premature) delivery and HIV-positive pregnancies are both regarded as high risk.

Preterm delivery may occur for a number of reasons (table 2.1) and the complications of prematurity may arise from adverse intrauterine or perinatal environments, physical size, or immaturity of body systems (Green and Morgan, 1993). In HIV-positive pregnancies there is a risk of vertical transmission of the virus from the mother to the baby which is highest during natural delivery. Antiretroviral drugs and caesarean section deliveries considerably reduce this transmission (Kind *et al.*, 1998). The lack of viral infection, however, does not preclude the neonate from immunological danger as the maturation of the immune system in uninfected neonates of HIV positive women is still compromised (Rich *et al.*, 1997).

Other predictors of high risk pregnancies may be certain medical conditions (diabetes mellitus, systemic lupus erythematosis, recent pulmonary embolism), previous obstetric history and cumulative risks of maternal age, parity, social class, smoking, attitude to pregnancy and acceptance of medical care (Johnstone, 1992).

1.1.10 Prematurity

Preterm birth is associated with higher incidences of perinatal mortality as well as short-term and long-term neonatal morbidity (Amon, 1999). The aetiology of preterm birth is varied (table 1.3) and may not always be predicted antenatally.

significantly more susceptible to infection than term babies (Lilja *et al.*, 1984). Complications of prematurity include respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), patent ductus arteriosus, necrotizing enterocolitis (NEC), hyperbilirubinemia, apnoea of prematurity, intraventricular haemorrhage (IVH), periventricular leukomalacia, retinopathy of prematurity (ROP) and neonatal sepsis (Amon, 1999).

Duggan *et al.* (2001) showed that in very preterm neonates (less than 32 weeks completed gestation) a consistent fetal inflammatory response and activation of immunological memory *in utero* is associated with cerebral damage presenting as intraventricular and periventricular lesions. Interleukin-6 (IL-6) a proinflammatory cytokine detected in maternal plasma of patients with preterm premature rupture of membranes, significantly ($p<0.0001$) predicted patients with neonatal complications for RDS, NEC, IVH and neonatal sepsis amongst others (Lewis *et al.*, 2001).

Although there appears to be no significant connection between BPD and infection such as chorioamnionitis with or without funisitis (Kent and Dahlstrom, 2004), preterm neonates at risk for the development of BPD show an enhanced inflammatory reaction in the lungs (tracheobronchial apirate fluid) and an associated increase in pulmonary microvascular permeability (Groneck *et al.*, 1994) the latter associated with the inflammatory response.

While prenatal brain injury may be associated with infection, it is difficult to argue that post-natal injury of the respiratory, neural and gastrointestinal systems is caused by infection as preterm infants are cared for under specifically aseptic conditions. There is clearly a need for better understanding of the developmental responses of the immune,

vascular and neural systems which are all comprised of highly responsive cell membranes involved in signalling recognition and physical response. Much work has already been done on the neural system in the interest of infant nutrition (Anderson and Maude, 1972; Neuringer *et al.*, 1984 and Farquharson *et al.*, 1992). However there is little published data on the development of the immune system and indeed the immune cells at birth.

1.1.10.2 The immunological status of babies that are born preterm

Generally in healthy infants the total number of T-lymphocytes increases from birth to later infancy (Raes *et al.*, 1993, Stern *et al.*, 1992 and O'Gormon *et al.*, 1998). This is especially noted in the CD4+/CD45RA+ cells (Raes *et al.*, 1993), CD3 cells and their T-cell subsets (CD4, CD8) (Stern *et al.*, 1992). The CD3+ lymphocytes increase 1.5 fold immediately after birth and decrease three-fold from two years of age until adulthood (Comans-Bitter *et al.*, 1997). CD3 is expressed on T- cells and is responsible for the T-cell receptor (TCR) signal transduction (Benjamini *et al.*, 2000). CD4 molecules are expressed by T-lymphocytes, thymocytes, monocytes and macrophages and are described as T-helper (Th) cells (Benjamini *et al.*, 2000). They give rise to Th1 and Th2 cells which express specific cytokines that are responsible for proinflammatory, autoimmune and anti-inflammatory responses. CD8 molecules are expressed on T-cell subsets and cytotoxic T-cells and are responsible as TCR co-receptors involved in signal transduction (Benjamini *et al.*, 2000). CD45RA (mainly in the CD4 population are markers for naïve lymphocytes), while CD45RO are memory T-lymphocyte markers (Table2.2) (de Vries *et al.*, 2000 and Neubert *et al.*, 2000). Neonate cord blood B and T-cells are immature (naïve), with virtually no "memory" cells which are abundant in adult blood (Neubert *et al.*, 2000).

According to Juretić *et al.* (2000), in comparison with term neonates (>37 weeks gestation), preterm neonates of a low gestational age (<32 weeks gestation) had lower percentages of CD3 T-cells of mostly the CD4+ subset. Furthermore, in preterm babies with early perinatal bacterial infection, the percentages of CD3+ T lymphocytes, CD4+ T-helper and CD8+ cytotoxic T-cells were lower compared to uninfected preterm neonates (Juretić *et al.*, 2001). However, the percentage of memory (CD45RO+) cells was also significantly lower than those of uninfected preterm neonates and were therefore not indicative of bacterial infection (Juretić *et al.*, 2001). The percentages of T-cells, cells bearing the T-cell receptors and natural killer cells were lower in fetal samples compared to term neonatal cord blood (Zhao *et al.*, 2002). There was also a negative correlation between CD4:CD8 T-cells between fetal blood and gestational age (Zhao *et al.*, 2002).

Meister *et al.*, 1994 found that the difference between term and preterm babies lay in the fraction of erythroid lineage CD34+ cells coexpressing CD71 antigens in preterm neonates compared to term. CD34+ molecules are markers for early stem cells (Benjamini *et al.*, 2000). The ratio of myeloid to erythroid lineage-specific progenitor cells, shows gestational changes in the lineage commitment of CD34+ cells (Meister *et al.*, 1994).

1.1.10.3 LCPUFAs and the preterm neonate

Babies that are born preterm are denied the placental nourishment that is usually awarded to babies born at term, and they are therefore exposed to higher oxygen tensions than normal (Crawford *et al.*, 1998). Preterm infants are more likely to be susceptible to peroxidative attack (mainly to proteins and PUFAs) due to a less highly

developed endogenous antioxidant enzyme system than that found in term babies (Phylactos *et al.*, 1995).

Peroxidation results in a loss of enzyme activity as well as the destruction of the cell membrane leading to leakage. The rupturing of the cell membrane leads to vasoconstriction and cell adhesion, and persistent damage would lead to infiltration, oedema and ischaemia (Crawford *et al.*, 1998). ROP is an oxygen-induced retinopathy and is associated with vascular cell injury culminating in microvascular degeneration (Hardy *et al.*, 2005). The retina is particularly rich in LCPUFAs. The ischemic retina is highly sensitive to lipid peroxidation initiated by oxygenated free radicals (Hardy *et al.*, 2005). In response to this two important lipid mediators are produced, the eicosanoids (such as thromboxane A_2) and platelet-activating factor. Although these mediators accumulate in the retina in response to injury, it is as yet uncertain what their physiological role is, however it has been postulated that they contribute to microvascular degeneration, endothelial cell death and the pathogenesis of ischaemic retinopathies (Hardy *et al.*, 2005).The effect of LCPUFAs on inflammation is seen in sepsis and the acute respiratory distress syndrome (Zaloga, 1999).

1.1.11 HIV and pregnancy

1.1.11.1 The Human Immunodeficiency Virus (HIV)

HIV is an enveloped retrovirus of the lentivirus family (Benjamini *et al.*, 2000). Two strains of the virus have been identified (HIV-1 and HIV-2), and of these HIV-1 is the more virulent (Benjamini *et al.*, 2000). The viral envelope is of critical importance in infection. It is primarily derived from the host cell membrane, but it displays viral glycoproteins such as gp120 and gp41 (Benjamini *et al.*, 2000). Gp120 has a high affinity for CD4 antigens and therefore cells expressing CD4 (including CD4+ T-cells)

are targets for the virus (Benjamini *et al.*, 2000). After binding to the CD4, gp120 must also bind to a co-receptor, which is CCR5 in macrophage tropic cells and CXCR4 in lymphotropic cells (Benjamini *et al.*, 2000).

The clinical course of HIV infection occurs in three stages. The first stage is that of acute infection and is characterised by a dramatic drop in the circulating CD4+ T-cells (Benjamini *et al.* 2000). The immune system responds by increasing the production of cytotoxic T-cells and antibodies specific for the virus. The patient has then "seroconverted" or has a detectable level of antibodies to the HIV protein, and the CD4+ count recovers (Benjamini *et al.*, 2000).

The second stage is the chronic latent phase. This is typically an almost an asymptomatic phase, although viral replication does occur at a lower level and the CD4+ count does slowly decrease (Benjamini *et al.*, 2000). Patients are governed at this stage by their CD4+ count and the CD4/CD8 ratio (where CD8 cells eventually outnumber the CD4 cells). As the CD4 cells reach progressively lower levels, the patient becomes increasingly symptomatic and enters the final crisis phase, acquired immunodeficiency syndrome (AIDS) (Benjamini *et al.*, 2000). The fall in CD4+ T-cell numbers leaves the patient in an immunodeficient state, and susceptible to opportunistic infections, and malignancies, and eventually death (Benjamini *et al.*, 2000 and Landers *et al.*, 1997).

1.1.11.2 HIV, pregnancy and vertical transmission

Pregnant women maintain immunocompetence throughout gestation, however woman tested positive for HIV (HIV+) may experience a decline in CD4+ lymphocyte number

and function (Landers *et al.*, 1997). Infection with HIV may predispose pregnant women to preterm labour, prematurity, lbw infants, postpartum endometritis and other infectious morbidity (Landers *et al.*, 1997).

HIV+ pregnant women are usually given an antiretroviral drug called azidothymidine (AZT) to decrease viral load and thus decrease transplacental transfer of the virus to the foetus. AZT (also known as zidovudine) is an inhibitor of reverse transcriptase contained within the viral core (Benjamini *et al.*, 2000). A second class of inhibitors often used are protease inhibitors, and more recently patients who are HIV+ but asymptomatic, have been given triple-agent antiviral therapy (with protease, reverse transcriptase and nucleoside inhibitors) (Benjamini *et al.*, 2000). Apart from placental transfer, passage through the birth canal and breastfeeding have also been implicated in mother to infant transfer of HIV (Benjamini *et al.*, 2000).

Two transmission patterns have been ascertained by Espanol *et al.* (1994) from 44 infected children as compared to uninfected children. These are: a) infants infected during pregnancy with severe immunodeficiency and clinical manifestations before 1 year old, and, b) children probably infected perinatally with better clinical outcomes.

1.1.11.3 Clinical complications of maternal HIV infection and pregnancy outcome

Infection with HIV may predispose pregnant women to the risk of preterm labour, low birth-weight neonates and intrauterine growth retardation (Goldstein *et al.*, 2000; Lambert *et al.*, 2000, and Castetbon *et al.*, 1999), as well as postpartum endometritis and other infectious morbidity (Landers *et al.*, 1997). Castetbon *et al.* (1999) attributed lbw in infants to low maternal weight as HIV+ women were found to weigh less than

HIV- women. Goldstein *et al.* (2000) show that women who received no treatment, zidovudine only and protease inhibitor treatment showed more lbw births than the comparison groups. HIV seropositive women also had high frequencies of several obstetrical risk factors for lbw infants (Goldstein *et al.*, 2000).

1.1.11.4 HIV and neonate immune parameters

CD4+ T-cell counts are significantly lower in infected infants at 3 months and 24 months (d'Arminio Monforte *et al.*, 1990). Serum Ig levels and CD8+ T-cell counts increase in infected children from 6 months (d'Arminio Monforte *et al.*, 1990). This is especially true of IgE levels, which increase significantly (Vigano *et al.*, 1995). The CD4+ T-cells and CD4/CD8 ratio decrease from 12 months of age (d'Arminio Monforte *et al.*, 1990).

CD8 T-cells are divided into naïve and memory cells, and in healthy infants there is a higher frequency of naïve cells and almost no memory cells (Rabin *et al.*, 1995). Using multiparameter flow cytometry to distinguish CD8 naïve T-cells (expressing CD11a, CD45RA and CD62L) Rabin *et al.* (1995) found that compared with HIV- children, the naïve subsets of CD8 T-cells in HIV+ children, decreased significantly with a concurrent increase in memory cells. Thus the total CD8 count remained unchanged. Therefore loss of naïve CD8 T-cells in HIV+ patients may contribute to the defects in cell mediated immunity, which worsens with disease progression and decreases in CD4 T-cell counts (Rabin *et al.*, 1995).

Rich *et al.* (1997) studied the immune parameters of uninfected infants born to HIV infected mothers. They found that the production of activated (CD4+HLA-DR+CD38+)

and memory (CD4+CD45RA-RO+) lymphocytes were higher in uninfected infants of HIV+ mothers than those of uninfected infants of uninfected mothers. Therefore the CD4+ lymphocytes of some HIV exposed, uninfected infants seem to have been stimulated at an early age (Rich *et al.*, 1997) meaning that the placental barrier may be permeable to microorganisms and proteins. Clerici *et al.* (2000) showed in uninfected neonates of HIV-positive women that the CD8 cell numbers were increased ($p<0.0001$), which meant that the CD4:CD8 ratio was decreased; naïve cells (CD4/45RA) was decreased while the memory cells were augmented ($p<0.0001$). The lower numbers of naïve cells was confirmed a year later by Nielson *et al.* (2001), when they found reduced thymic output in uninfected neonates which together lower neonatal red blood cell counts, suggested impaired progenitor cell function. Importantly, Clerici *et al.* (2000) also found that in uninfected neonates of HIV positive mothers, HIV-specific CD4 T-cells were strongly present and the responded to the viral envelope during incubation, but were not responsive to the influenza virus. The HIV exposed T-cells of the uninfected neonates was also very responsive to mitogen stimulation, but not so the healthy controls (Clerici *et al.*, 2000). It has also been shown that the frequency of HIV-specific $CD4^+$ T-cells was augmented in HN neonates, but that HIV-specific interferon-gamma secreting CD8 T-cells were present in only a small number of individuals, which indicated that intrauterine exposure of HN neonates may have been subsequent to transplacental diffusion of HIV soluble proteins and not to live and replicating viruses (Nielson *et al.*, 2001). Others have shown that the cord blood T-lymphocytes of seroreverter (uninfected neonates born to HIV-positive women) neonates are more susceptible to apoptosis (Economides *et al.*, 1998). For simplicity, the term definitely not infected (DNI) has been used to describe uninfected neonates of HIV-infected women. This term had originally been adopted in lieu of "seroreverter" by the Centre for Disease Control (CDC) and the Indiana State Department of Health[5].

1.1.11.5 LCPUFAs and HIV

A major complication of the course of HIV infection is that of malnutrition, which although more prevalent in developing countries, did not altogether disappear in Western countries, particularly in patients with long-term infection and a history of treatment failures (Newell *et al.*, 2004). Malnutrition does not simply refer to starvation and the loss of body fat, but rather to the loss of lean body mass due to wasting as well as deficiencies in micronutrients such as vitamin A. Studies show that vitamin A deficiencies are associated with mother-to-child transmission of HIV (Semba *et al.*, 1994) and that supplementation with multivitamins improve pregnancy outcome as well as increase T-cell counts in HIV positive women (Economides *et al.*, 1998; Fawzi *et al.*, 1998). Although long chain polyunsaturated fatty acids (LCPUFA) are known to influence immune function, not much is known about how deficiencies in these super-unsaturated fatty acids may influence the immunological state of an uninfected neonate born to an HIV-infected mother. In HIV-infected children, changes in the plasma fatty acid profile indicates increased LCPUFA turnover (Agostoni *et al.*, 1998). These changes in LCPUFA composition may be of relevance to the regulation of inflammatory responses in the neonates.

Although not much is known about the specific association between LCPUFAs and the neonatal immune system status in prematurity and low birth weight (irrespective of the cause), there is a compelling case for the involvement of LCPUFAs.

1.1.12 Thesis objectives

As stated previously, the objectives of this project were to examine the fatty acid composition of the major phosphoglycerides of cord blood mononuclear cell membranes of healthy term, preterm and DNI neonates. In addition the cord blood

immunophenotype and subset numbers were analysed by flow cytometric methods using immunolabelling. Based on the data obtained further work was conducted in determining the response of term, preterm and DNI CBMCs upon culturing in media containing exogenous unesterified-AA.

If dietary deficiencies could be tangibly connected to poor immune system function, it would suggest antenatal LCPUFA supplementation may be beneficial, particularly with respect to health care in the developing countries where maternal and fetal/neonatal wellbeing is compromised by opportunistic and chronic infections, particularly in cases of HIV-infection.

Chapter 2

2.0 Materials and Methods

2.1 Patients and sample collection

Healthy women delivering at term (37-40+ weeks gestation), preterm (30-36 weeks gestation) and HIV-positive women delivering at term were recruited at Newham University Hospital, London. The women were aged between 20-40 years with a parity of 0-3. Prior to delivery women in preterm labour were treated with dexamethasone sodium phosphate (dexamethasone, 12mg); a majority of women (n=14) received a second dose approximately 12 hours after the first dose. This data is shown on table 2.1 with other relevant data concerning the mothers and their babies.

All of the HIV-positive women enrolled in this study were on anti-retroviral prophylaxis (ARV) (Table 2.1). None of the neonates born to HIV-positive women were infected with HIV as detected by PCR at 4 weeks, 3 months and 6 months old and were therefore referred to as definitely not infected (DNI)[5]. All the HIV-infected mothers were administered ARVs from a minimum of 4 hours prior to delivery by caesarean section. In all cases, zidovudine (AZT) was administered until the umbilical cord was cut. A majority of HIV-positive women (n=6) were given AZT during pregnancy, while a minority of cases (n=3) were given alternative ARV prophylaxis according to stage of disease; a combination of combivir and nevirapine was used. The newborns were given AZT orally after delivery. Cord blood was collected after informed consent was given. This study was approved by The East London Health Authority Ethics Committee (approval number N/99/085) and cord blood samples were collected only after informed consent was given.

2.2 Flow Cytometry

Cytometry is the measurement of physical and chemical characteristics of cells and other biological particles (Shapiro, 1995). Flow cytometry (flow cytofluorimetry) is an immunofluorescence technique that analyses populations of single cells by their cell surface membrane antigens (Polak and Van Noorden, 1997). The cells are separated on the basis of their fluorescence and light-scattering abilities (dependent on cell size and internal morphology) (Johnstone and Thorpe, 1996 and Polak and Van Noorden, 1997). The cells in suspension are passed singly and rapidly through the focus of a laser beam, (Shapiro, 1995) and the fluorescence of the cells can be assessed and quantified (Polak and Van Noorden, 1997).

A flow cytometer records several parameters for each cell including forward scatter, side scatter and fluorescence intensity (Johnstone and Thorpe, 1996). Particles that are relatively small with homogenous internal structure scatter (reflect) light at small angles to the incident beam producing what is commonly known as forward or small angle scatter (Shapiro, 1995). Particles that have complex surfaces or granulated internal structure, tend to scatter more light at larger angles producing what is commonly referred to as right-angle or side scatter (Shapiro, 1995). Fluorescently-labelled antibodies to cell surface makers provide an instantly visible fluorescent label (Polak and Van Noorden, 1997). The fluorescent particles have different excitation and emission wavelengths (shown in table 2.2) and are viewed with the use of separate filter systems.

2.2.1 Setting up the flow cytometer and quality control

In order to ensure that the results obtained were accurate and unbiased several tests were to run prior to sample testing on the flow cytometer. There were three main controls that

were run to correct counting errors. Errors may occur due to the following (Longobardi Givan, 1992):

- The presence of unstained cells (background fluorescence)
- The nature of antibody-antigen interactions or non-specific binding
- Overlapping fluorochrome spectra (the compensation problem)

2.2.1.1 Istotype controls

Some cells may auto fluoresce as a result of intracellular constituents such as flavins or pyridine nucleotides, while others may possess surfaces that attach any fluorescently conjugated antibody (Longobardi Givan, 1992). The isotype control is an antibody with the same properties of the antibody of interest, i.e. with the same immunoglobulin subclass and number of attached fluorescein labels, but without specificity for the antigen (Longbardi Givan, 1992). This enabled the detection of auto fluorescing cells as well as non-specific cell staining. The appearance of these cells was then corrected on the flow cytometer by adjusting the voltage of the laser, thereby keeping the isotype controls (and in the actual samples the non-specific stained cells) in the first log decade of the histogram (figure 2.1).

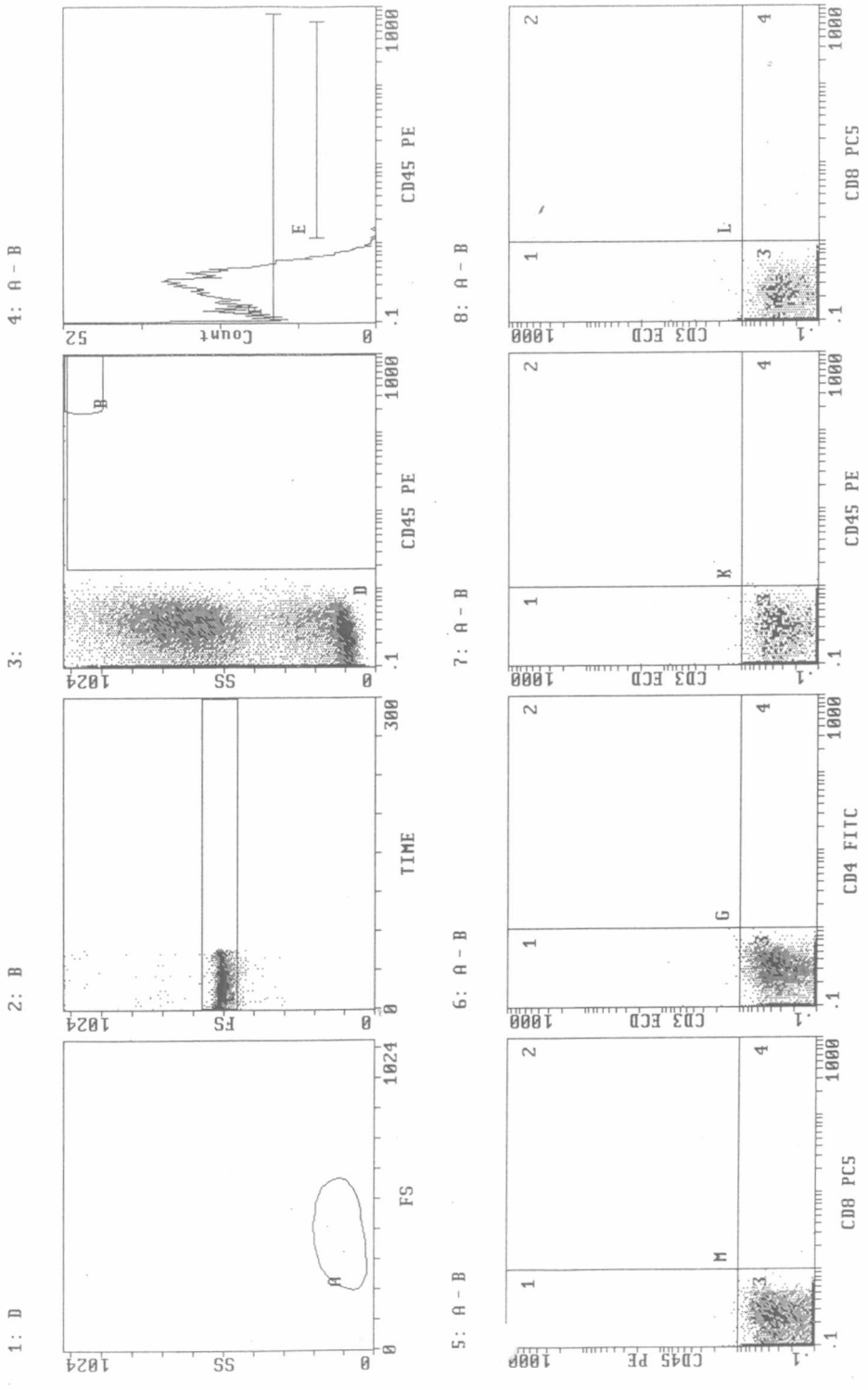


Figure 2.1: Histogram showing the isotype control stained cells.

2.2.1.2 Compensation of spectral crossover

Each fluorescent colour was emitted via its own filter which had a specific wavelength range. This is illustrated in figure 2.2, as a means of explaining the crossover of the spectra which are detected by the filters. In this case the flow cytometer contained four spectral filters and was therefore named the XL-4 flow cytometer (Beckman Coulter). However as a result of the emission wavelengths being fairly similar there was a chance that cells of a specific colour may be detected in the wrong filter and this is shown as the pink, blue and green overlapping areas in figure 2.2. Therefore each of the fluorescent antibodies had to be run and the filters compensated individually and eventually in pairs in order to ensure that crossover did not occur. This was achieved by adjusting the voltage of each filter and these voltages were maintained for the remainder of the analytical process (figure 2.3). The cell samples with their conjugated antibodies were then gated for the lymphocyte population and the programme template was saved so that the same settings could be used for subsequent analyses.

2.2.1.3 Quality Control

At the beginning of every new session with the flow cytometer it was important to check the optical alignment and fluidity. Cell debris within the machine could affect the results. Polystyrene Quantum Flow-Check beads (Beckman Coulter) in suspension in an aqueous buffer were used to assess the functioning of the machine. Histograms outlining the half-peak coefficient of variation (half-peak CV) were produced for each filter (figure 2.4). The half-peak CV had to be less than 2 in order for a filter to pass inspection. If a filter did not pass inspection the cleanse and prime cycle were run and the half-peak CV was reassessed.

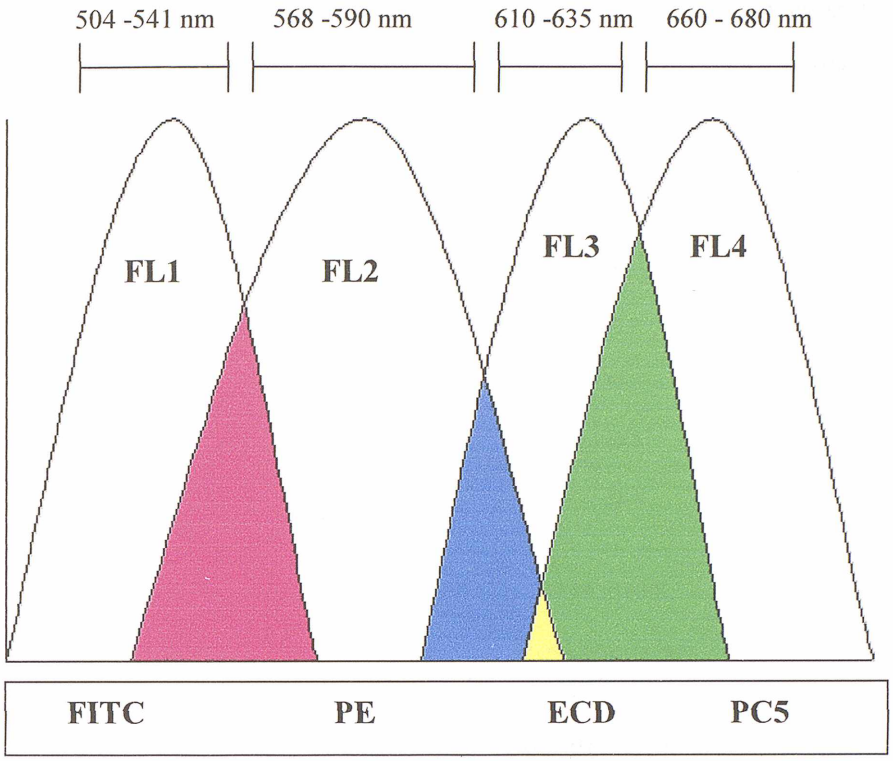


Figure 2.2: **Spectral filter crossover of wavelengths**. The pink, blue, yellow and green areas of the figure show the overlapping of spectra for the four filters (FL1-4). Where FITC is fluorescein isothiocyanate; PE is phycoerythrin; ECD is phycoerythrin-Texas Red and PC5 is phycoerythrin-cyanin. Therefore if the FITC is leaking into the PE filter, then the PE filter must be adjusted using the voltage setting for FL2 to remove the FITC emission. This was visualised in the histograms as the cells became localised within their own filter quadrants.

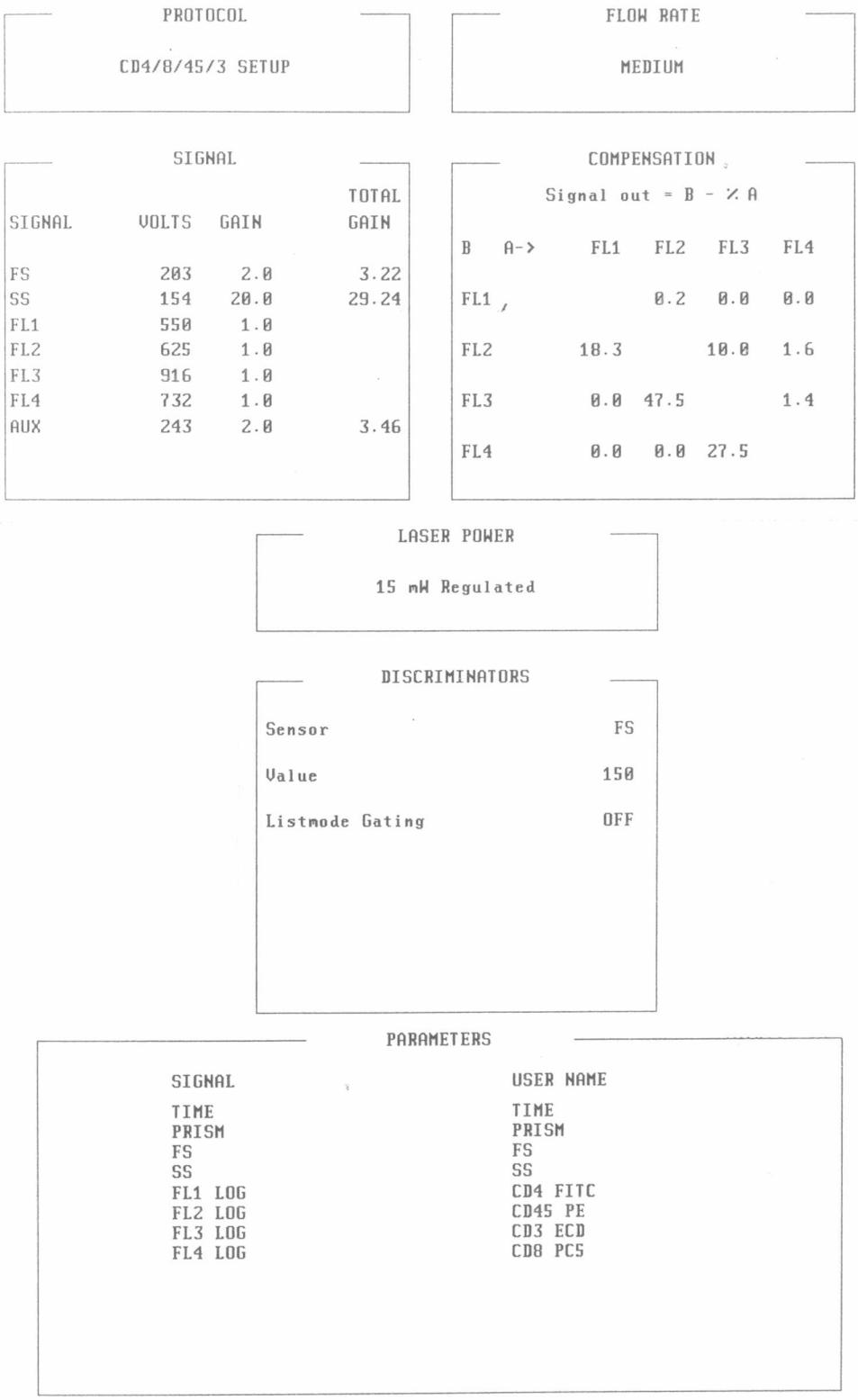


Figure 2.3: Epic XL (Beckman Coulter) flow cytometer settings printout. Where FL is filter, FS is forward scatter, SS is side scatter and GT is gate.

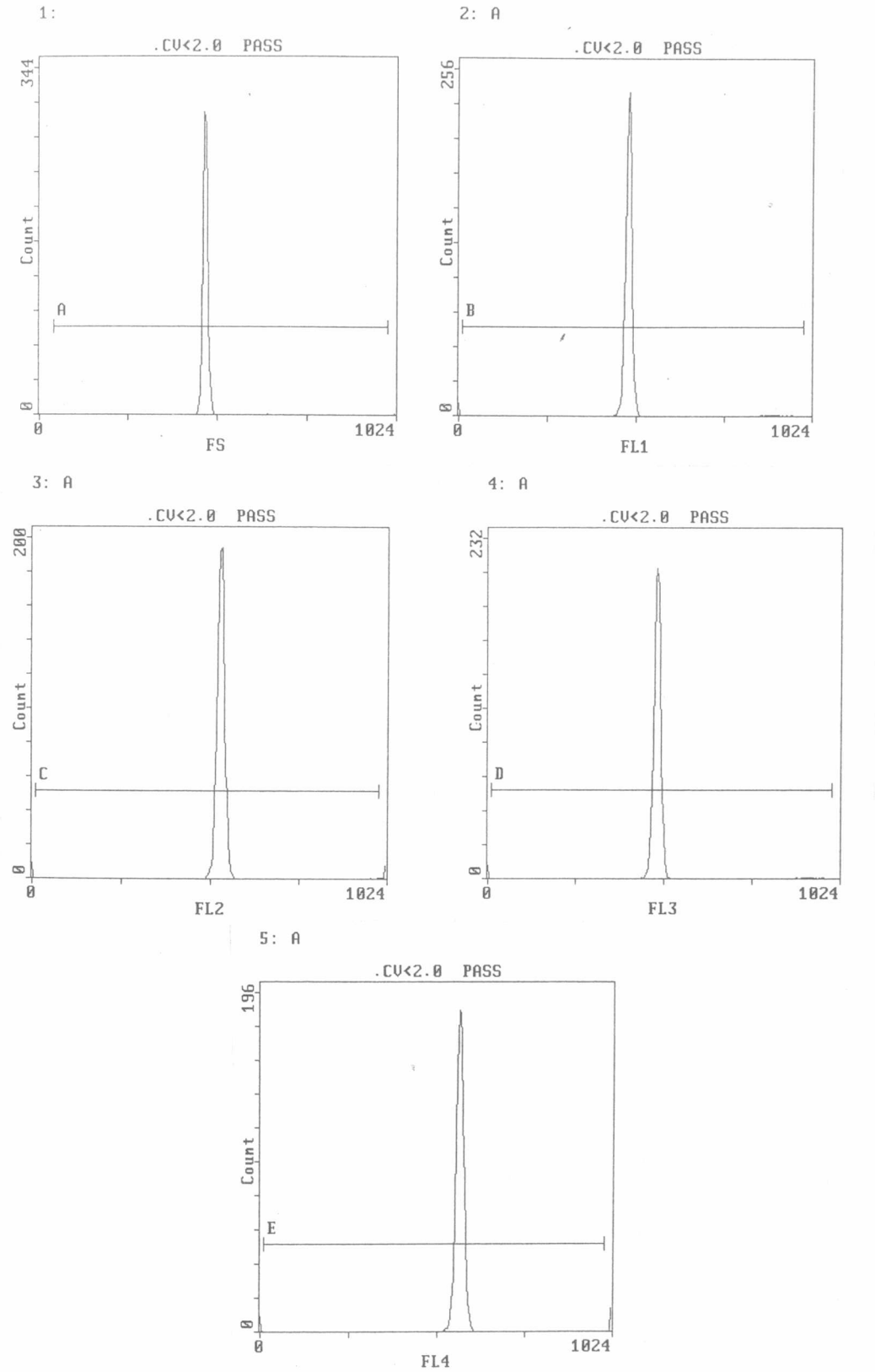


Figure 2.4: Coulter acquisition flow cytometry quality control report. This report shows the results of the quantum flow check, where CV<2 is coefficient of variation less than 2, which indicated that the lasers and filters passed the quality control test.

2.2.2 Processing of whole cord blood samples for flow cytometry

The need for immunological references of both an identification and functional nature is important clinically. It is also of importance to note whether the reference values for paediatric immunology can be used to diagnose and possibly treat patients with haematologic and immunologic disorders. These reference values are gathered by immunophenotyping and sorting the various cells of the immune system. This is usually achieved by analysing the clusters of differentiation (CD) antigens, which are expressed by various cells in the immune system (Benjamini *et al.*, 2000). These cell surface markers are associated with distinct developmental stages and important biological functions (Benjamini *et al.*, 2000).

Prior to analysis, 100µl of whole cord blood was incubated with combinations of fluorochrome-conjugated monoclonal antibodies (IOtest conjugated monoclonal antibodies; Beckman Coulter) raised against lymphocyte cluster of differentiation (CD) antigens (table 2.2) for 10 minutes in the darkened Q-Prep carousel (Beckman Coulter – figure 2.5) at room temperature. The stained whole blood sample tubes were then presented into the reagent dispense head sequentially. Red blood cells were lysed and white blood cells were fixed in the Q-prep machine using ImmunoPrep Solutions A (erythrocyte lytic agent), B (leukocyte stabilizing buffer) and C (cell membrane fixative) (ImmunoPrep Reagent System, Beckman Coulter). The use of the Q-Prep machine reduced contamination and the variability that can be introduced during manual processing.

Flow-count fluorospheres (Beckman Coulter) in an aqueous suspension medium containing 1% formaldehyde when added to the lysed and fixed whole blood was used to calculate the absolute count of cells per microlitre of fluid. 100µl of the 10µm (diameter) polystyrene fluorospheres (Beckman Coulter) were added to each tube. The

mixture was vortexed and the tubes were then placed individually within the receptacle of the flow cytometer and the listgate was analysed at 25000 events per sample. Each event represented one cell. Since the concentration of the fluorospheres was known, this value was added into the calibration programme of the Epics XL, and the absolute count of cells was automatically calculated using the following formula (Beckman Coulter Product Information Leaflet):

Absolute count (cells/μl) =

$$\frac{\text{Total number of cells counted}}{\text{Total number of fluorospheres counted}} \times \text{Flow-Count Fluorospheres assayed concentration}$$

Isotype controls (table 2.2, figure 2.1) were run for each antibody in order to identify background and non-specific staining. Lymphocyte subsets present in fresh whole cord blood samples tubes were analysed by four-colour flow cytometry with an EPICS XL-4 flow cytometer (Beckman Coulter – Figure 2.6) and the raw data was presented on flow cytometric histograms (figures 2.7 to 2.9 discussed below).

2.2.3 Identification and quantification of immune cell subset numbers

Figures 2.7-2.9 show the gating of the fluorochrome conjugated cells as they appeared on the Epics-XL4 (Beckman Coulter) run histograms. Each run required 8 histograms. The first histogram showed the gating of lymphocytes in region A (figures 2.7-2.9). Lymphocytes are depicted as being separated from neutrophils and other granulocytes by forward and side light-scatter properties (section 2.2). The second and third histograms show the flow and counting (region B) of the flow-count fluorospheres used to calculate the absolute count of each subset (methods section 2.2.2). The fourth histogram shows the count of all cells that are labelled with CD45 (the leukocyte marker). The remainder of the histograms shows a combination of markers determining

the subset groups, ie. cells that are specifically T-cells (CD3+) or cells that are naïve (CD45RA+). Histograms 5 to 8 are separated into quadrats. In figure 2.7 for example, quadrat 1 in histogram five would depict the number of cells that were stained with only CD45-PE, while quadrat 4 depicts the number of cells stained with only CD8-PC5. Quadrat 3 depicts dead cells and debris. The quadrat of interest is quad 2 which shows cells that are CD45+ and CD8+ (double stained).

Table 2.2: Monoclonal Antibody Combinations for Cord Blood Mononuclear Cell Analysis

Flourochrome	**FITC**	**PE**	**ECD**	**PC5**
(µl/100µl whole blood)	20µl	20µl	10µl	10µl
Excitation wavelength (nm)	468-505	486-575	486-575	486-580
Emission wavelength (nm)	504-541	568-590	610-635	660-680
Isotype controls	IgG1 (679.1Mc7)*	IgG1	IgG1	IgG2a (U7.27)*
T-cell CD marker	CD4 (13B8.2)*	CD45 (J33)*	CD3 (UCHT1)*	CD8 (B9.11)*
B-cell CD marker	CD19 (J4.119)*	CD45	CD3	CD25
Memory/naïve CD marker	CD4	CD45RA (ALB11)*	CD45RO (UCHL1)*	CD8

Where: FITC= fluorescein isothiocyanate; PE= phycoerythrin; ECD= phycoerythrin-Texas Red, PC5= phycoerythrin-cyanin 5.1, IgG= immunoglobulin; CD= cluster of differentiation; CD4 (T-helper cells); CD3 (Pan T-cells); CD8 (cytotoxic T-cells); CD25 (activated/ interleukin-2 producing cells); CD45 (Pan leucocytes); CD19 (B-cells); CD45RA (naïve cells); CD45RO (memory cells), * Beckman Coulter clone number.

Figure 2.5: The Q-Prep machine used for the staining, lysing and fixing of cells. The carousel underneath the darkened cover (C) housed 32 tubes at a time. The control panel (P) was used to set the incubation time and initiate the lysing and fixing process. The Q-Prep automatically began the lysing, buffering and fixing sequence of each tube after the incubation.

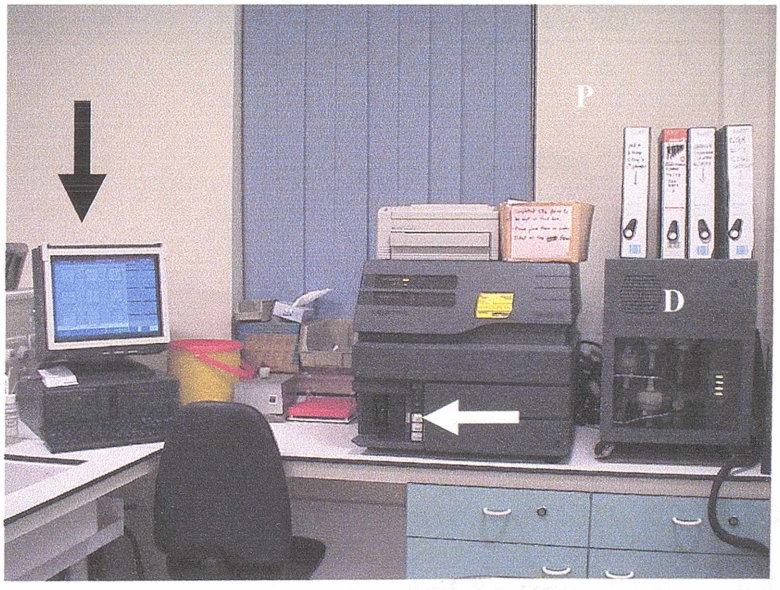


Figure 2.6: The flow cytometer consisted of a PC (black arrow), with built in epics XL software. The flow cytometer itself with a manual sample feed station (white arrow) and a drainage system for waste material and chemicals (D).

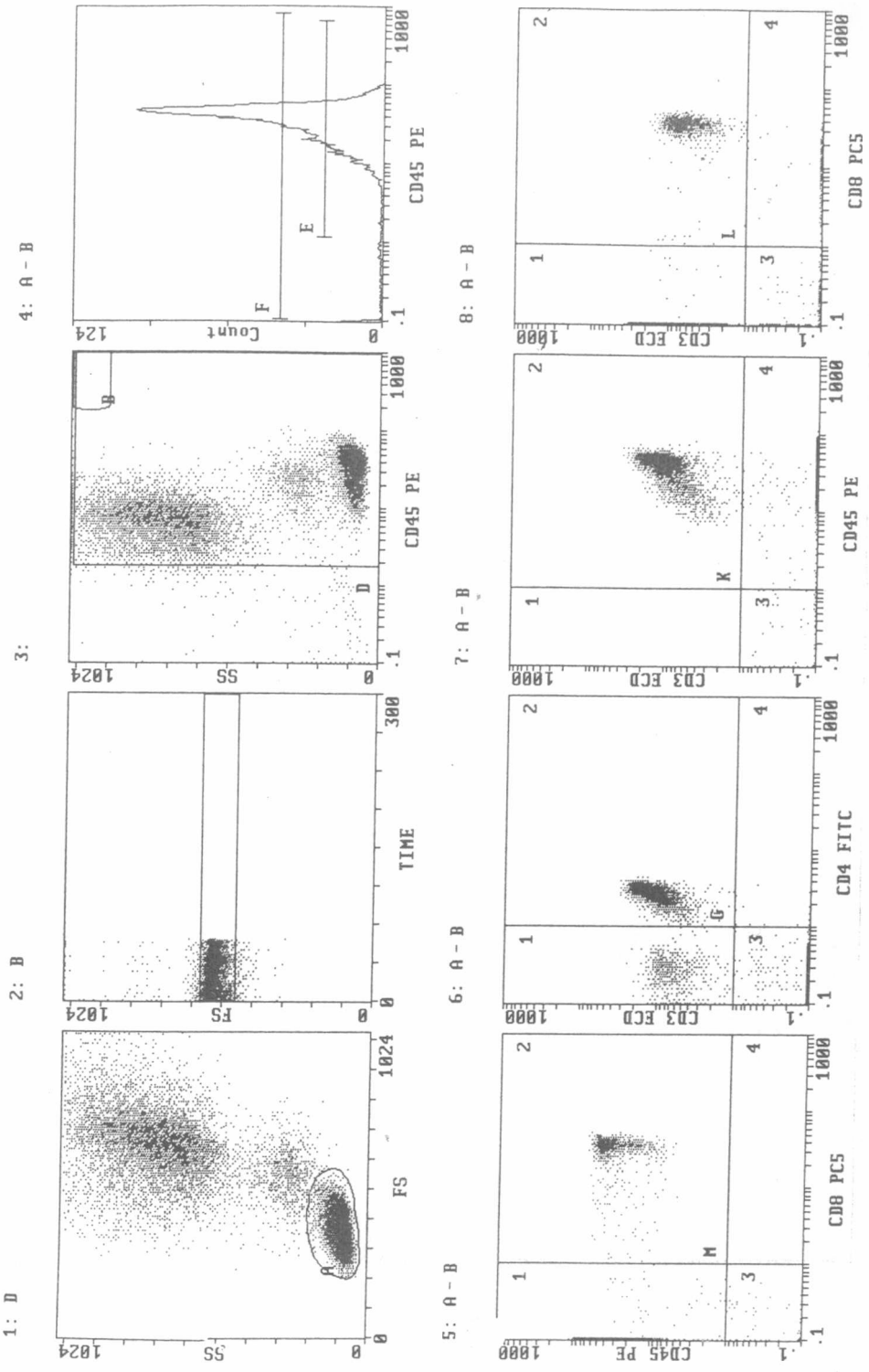


Figure 2.7: The histogram of CBMCs stained with CD3, CD4, CD8 and CD45 antibodies conjugated to ECD, FITC, PC-5 and PE respectively. Gated CD45+ cells are shown in histogram 1 (region A). The gating of the flow count fluorospheres is shown in histograms 2 and 3 (region B). The remainder of the histograms shows positively stained cells in regions E, M, G, K and L.

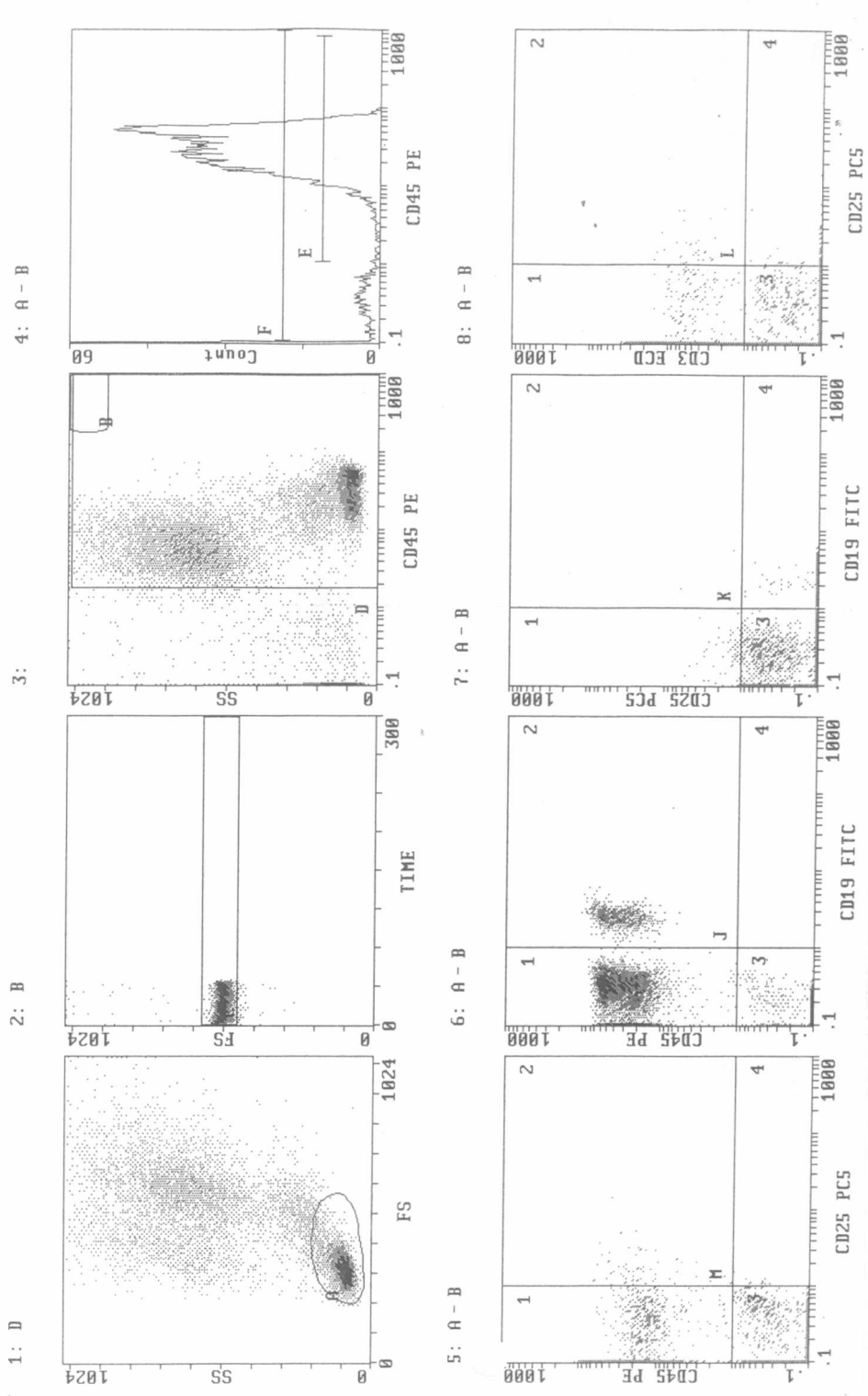


Figure 2.8: The histogram of CBMCs stained with CD3, CD19, CD25 and CD45 antibodies conjugated to ECD, FITC, PC-5 and PE respectively. Gated CD45+ cells are shown in histogram 1 (region A). The gating of the flow count fluorospheres is shown in histograms 2 and 3 (region B). The remainder of the histograms shows positively stained cells in regions E, M, G, K and L.

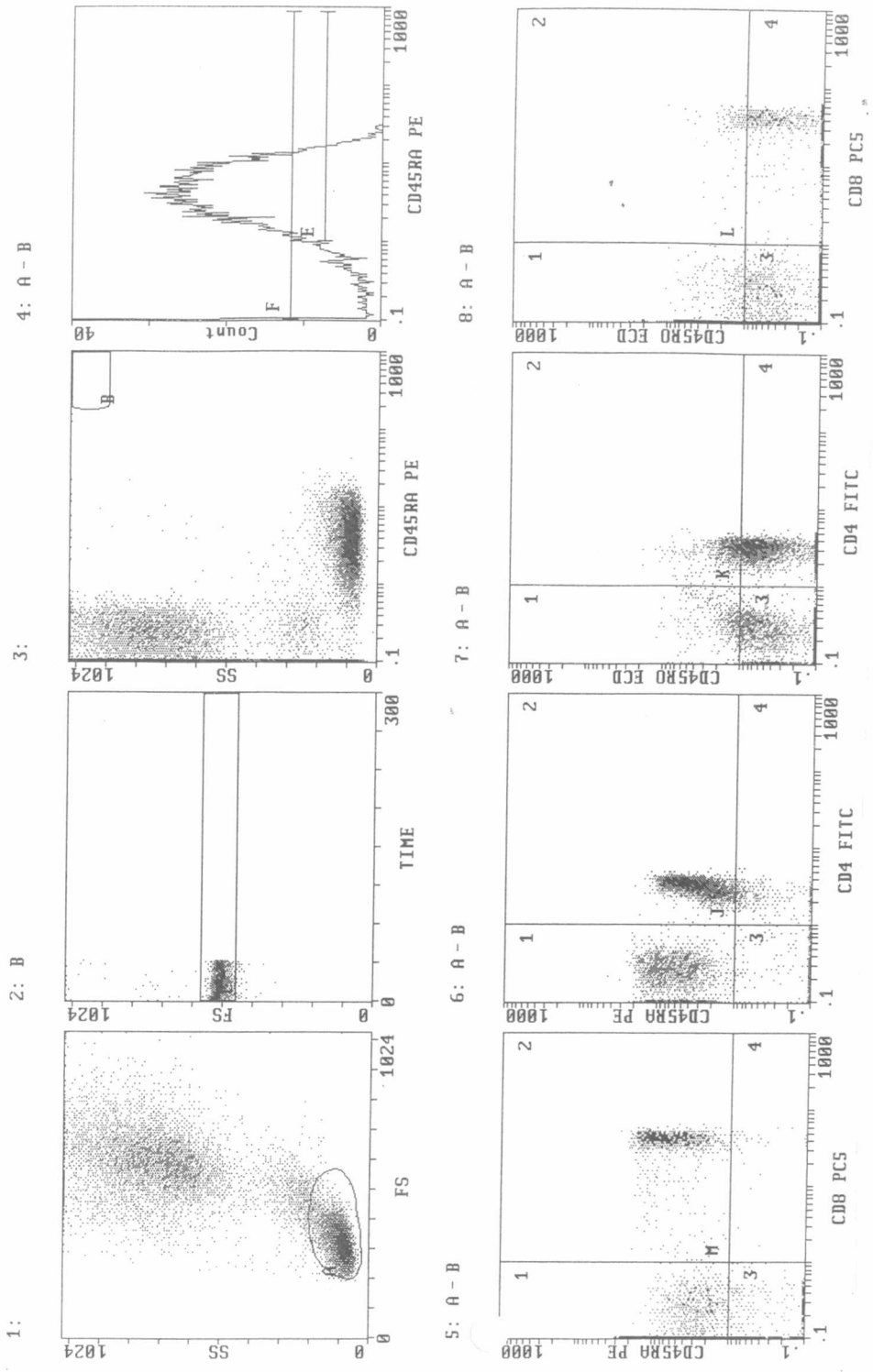


Figure 2.9: The histogram of CBMCs stained with CD4, CD8, CD45RA and CD45RO antibodies conjugated to FITC, PC-5, PE and ECD respectively. Gated CD45+ cells are shown in histogram 1 (region A). The gating of the flow count fluorospheres is shown in histograms 2 and 3 (region B). The remainder of the histograms shows positively stained cells in regions E, M, G, K and L.

2.3 Preparation of lymphocyte samples

In preparation for the gas chromatography and proliferation studies, cord blood mononuclear cells (CBMC) had to be isolated from the whole cord blood. Cord blood mononuclear cells comprise of lymphocytes and monocytes. All the isolation procedures were carried out under sterile conditions in a cell culture laboratory that contained a laminar flow cabinet and a sterile CO_2 incubator, to minimize damage and contamination of the cells particularly cells that were isolated for proliferation studies.

Prior to each session hands were washed with Hydrex surgical scrub. This served to sterilise and moisten the hands and thereby reduce the risk of dry skin blowing onto the cultures as well as reduce loosely adherent micro-organisms which are the greatest risk to the cultures (Freshney, 1983). Sterile gloves were thereafter used and swabbed intermittently with 70% ethanol and 30% methanol. The laminar flow cabinet was swabbed down with 70% ethanol and 30% methanol prior o and after use.

2.3.1 Extracting umbilical cord blood

Blood was taken under vacuum into 5ml EDTA tubes from the umbilical vein immediately after the placenta was delivered. The umbilical cord attached to the placenta was clamped at the cut end to ensure that whenever possible as much blood as possible was collected for flow cytometry and gas chromatography. When larger amounts of blood >20ml could be collected from the cord vein and venules, then these samples were used for proliferation studies.

2.3.1.1 The Kleihauer Method

The Kleihauer method (Shepard's Modification) is a standard method routinely used in Newham University Hospital NHS Trust, to detect fetal haemoglobin (haemoglobin F)

in maternal cells. The Kleihauer method is used to elute out adult red blood cells which then appear as white ghost cells, while white blood cells stain greyish-purple and cells with haemoglobin F stain red. The reagents (Clin-Tech, Essex) consist of alcoholic haematoxylin and ferric chloride. Briefly the method includes preparing a smear of the sample blood which was then air-dried and fixed in 80% ethanol (EtOH) for 5 minutes at room temperature. The slides were placed in the elution solvent [Alcoholic haematoxylin: acidified ferric chloride: 80% EtOH (2:1:1)] for approximately 20 seconds, and thereafter rinsed under cold running water. The slides were then placed into eosin counterstain for 2 minutes and rinsed in cold running water. The stained smears were examined with oil immersion microscopy at x500 magnification.

The objective of these tests was the opposite of that carried out in haematology laboratories as in this case the presence of maternal cells was looked for in fetal circulation. Therefore it was hoped that only fetal cells (stained red) would be seen. This was seen in all the cord blood samples and an example of a Kleihauer stained slide is shown in figure 2.10, where only stained red cells (fetal cells) are seen.

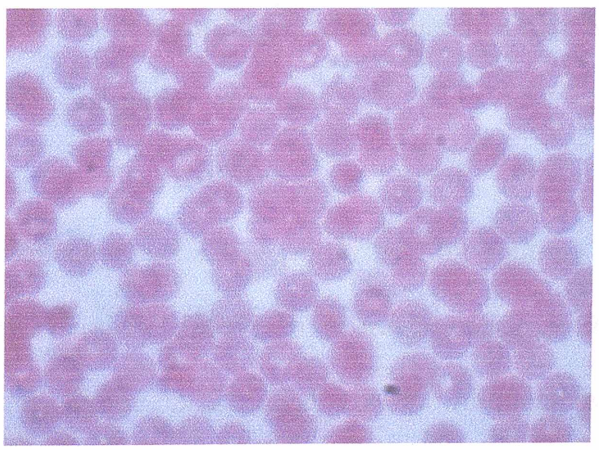


Figure 2.10: Cord blood red cells stain red with the Kleihauer reagents and confirm that maternal cells (white ghost cells) in which the adult haemoglobin is eluted out.

2.3.2 Isolation of Mononuclear cells from cord blood

CBMCs were isolated using density gradient centrifugation using Histopaque 1077 (Sigma). Histopaque 1077 is a solution of polysucrose and sodium diatrizoate with a density of 1.077 ± 0.001g/ml (Sigma procedure leaflet).

Whole cord blood was layered on equal volumes of Histopaque 1077 (room temperature) in 10ml sterile polystyrene tubes and centrifuged at 400xg for 30 minutes. This allowed the erythrocytes and granulocytes to sediment to the bottom of the tube (figure 2.11). The buffy coats (containing lymphocytes and monocytes) were aspirated with sterile glass pipettes and relayered on Histopaque 1077 and centrifuged as before. This was done to reduce erythrocyte and nucleated erythroid precursor contamination with no effect on lymphocyte subset numbers and function (Yang and Lin, 2001).

The final buffy coat preparations were aspirated and suspended with physiological saline (pH 7.4) in a fresh tube. The suspensions were centrifuged at 250xg for 10 minutes. The supernatants were aspirated and discarded and the resultant CBMC pellets were resuspended in physiological saline (pH 7.4) and centrifuged at 250xg for a further 10 minutes. This procedure was repeated twice more in order to remove extraneous platelets from the CBMC population of cells.

The isolation of mononuclear cells was confirmed by staining CBMC smears with Wright's stain (Hematek) and the slides were observed under oil immersion microscopy at 500x magnification (figure 2.12). The CBMC aliquots were used fresh for proliferation studies but were frozen at -20^{o}C until processing for fatty acid analysis of both inner and outer membrane leaflets.

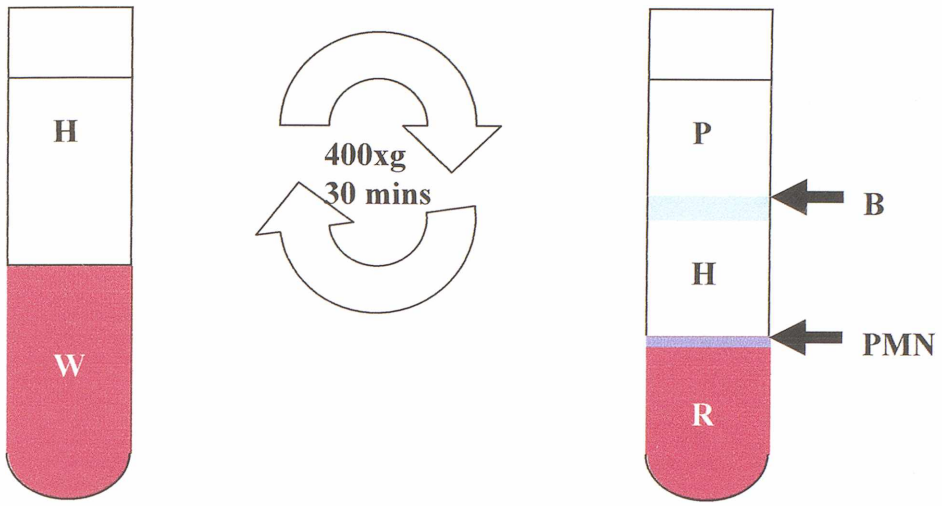


Figure 2.11: Depicting the density gradient centrifugation at 400xg for 30 minutes of whole cord blood. Where W is whole cord blood, H is Histopaque 1077, P is plasma, B is buffy coat containing lymphocytes and monocytes, PMN is polymorphonuclear leukocytes comprising of neutrophils and other granulocytes, and R is sedimented red blood cells.

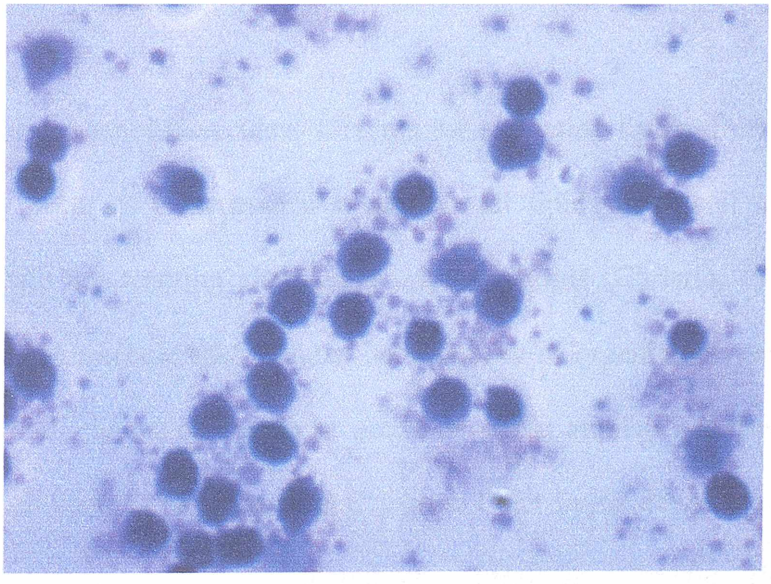


Figure 2.12: CBMC smears on glass slides stained with Wright's stain, shows the presence of lymphocytes of different sizes.

2.4 Fatty acid analysis

The fatty acid analysis of the isolated CBMCs was carried out by extracting and purifying lipid from cell membranes of stored CBMCs. The lipid was then separated into phospholipid groups and these were methylated and analysed using a gas chromatograph. The process is outlined below.

2.4.1 Extraction of lipids from CBMCs

The samples stored at -20°C were defrosted in a darkened room to prevent irradiation and consequent oxidative damage to the cells.

The cells were transferred to 50ml extraction tubes to which was added a mix of chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxy toluene under oxygen-free nitrogen (Folch *et al.*, 1957). The solution was capped and vigorously shaken in a fume cupboard. BHT was used as an antioxidant due to the ability of LCPUFAs to rapidly auto oxidise (Christie, 1982).

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Chloroform and methanol were ideal solvents for extracting lipids from tissues as they are sufficiently polar as is necessary to remove all lipids from cell membranes and lipoproteins while not reacting chemically with the lipids (Christie, 1982). The main features of a lipid molecule that dictates its solubility in organic solvents are the non-polar hydrocarbon chains of the fatty acids or other aliphatic moieties, and polar functional groups such as phosphates and sugars. Polar lipids dissolve readily in more polar solvents such as methanol, ethanol or chloroform (Christie, 1982). A blank extraction tube was prepared with 15ml of methanol and 30ml of chloroform. The extractants and blank were then flushed with nitrogen gas and immediately capped to prevent oxidation of the lipids via the entry of air into the tube. Care was taken to flush gas wand in chloroform methanol and wipe it clean before introduction into the each

tube prior to flushing the contents with nitrogen. This was done to prevent sample to sample contamination. The extraction tubes were then placed at 4°C overnight to allow extraction to occur.

2.4.2 Partitioning of the solvent and aqueous phases

The contents of the extraction tubes were filtered into glass-stoppered separating funnels with clean Teflon taps. The filter consisted of clean filter paper (Whatman). The extraction tubes were rinsed with 2 x 10ml washes of chloroform methanol (2:1) with BHT and poured into the filter of the separating funnel. Saline (0.85%) at 25% v/v of the contents of the separating funnel was introduced and constituted the upper cleaning phase. Care was taken to use gloves the apparatus that neither the Teflon taps, nor and the filter paper to avoid contamination. Each separating funnel was flushed with nitrogen gas as before, and immediately capped and stored overnight at 4°C.

2.4.3 Rotor Evaporation

The lower phase of the partition (the solvent) in the separating funnel containing the lipids were extracted into round bottom flasks. The solvents were removed from the lipid extracts under vacuum using a rotor evaporator. There was no need for a constant stream of nitrogen gas during this evaporation as the solvent vapours displaced any air (Christie, 1982).

2.4.4 Thin Layer Chromatography (TLC)

The method required a polar solvent system and in this case a mixture of Chloroform, methanol and methyl amine (volume ratio of 65:35:15) was used. The tanks were rinsed with a small volume of the same solvent prior to use. Approximately 200ml of the solvent was placed in the tank which was lined with filter paper. The tanks were sealed and allowed to equilibrate for 30 minutes.

The TLC plates (Whatman Silica plates; K6 silica gel 60Å -20x20, 250μm thickness) were baked in the 100°C oven for 1 hour and placed in the desiccator for a further hour to ensure that they were thoroughly dry.

The samples were loaded on the TLC plates using capillary tubes at a maximum of 15-20mg per sample (Henderson and Tocher, 1992). The plates were carefully placed in the tanks which were sealed, and left for an hour to separate. Thereafter the plates were removed and dried and sprayed with 2,7 dichlorofluorescein in methanol (0.1% solution) to identify the phospholipids bands. The bands were visualised under UV light in the darkroom and could be distinguished with their positions on the plate (Figure 2.13) as well as corresponding in-house standards.

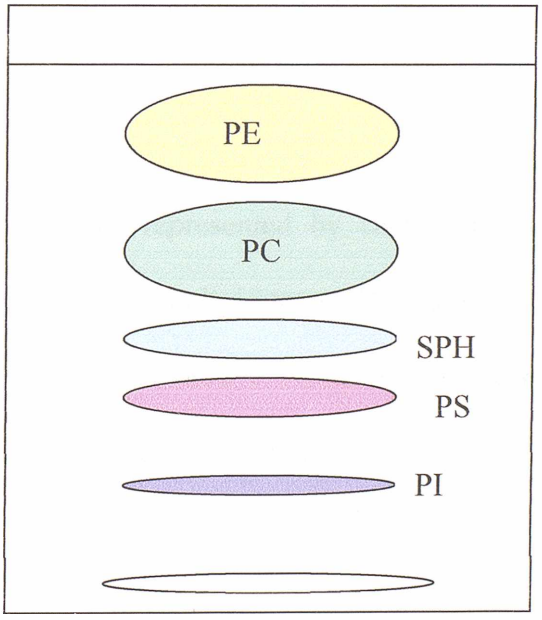


Figure 2.13: An example of a TLC plate with the separated phospholipids where: PE is phosphatidyl ethanolamine, PC is Phosphatidyl choline, SPH is sphingomyelin, PS is Phosphatidyl serine and PI is phosphatidyl inositol.

2.4.5 Methylation of fatty acids

The fatty acid methyl esters (FAMEs) of each phospholipid band were prepared by heating lipid fractions in 4ml of 15% acetyl chloride in methanol at 70°C for 3 hours under oxygen-free nitrogen.

2.4.6 Analysis of fatty acids by gas chromatography

FAMEs were separated in a gas liquid chromatograph (HRGC MEGA 2 Series, Fisons Instruments, Italy) fitted with a capillary column (25m x 0.32mm ID, 0.5μ film, BP20). Hydrogen was used as a carrier gas, and the injector, oven and detector temperatures were 250°C, 200°C and 280°C respectively. FAMEs were identified by comparison of retention times with authentic standards and calculation of equivalent chain length values. Peak areas were quantified by a computer chromatography data system (EZChrom Chromatography Data System, Scientific Software, Inc., San Ramon, CA). An example of the chromatography histograms for a term and preterm neonate are shown in figure 2.14.

The area under the peaks represented by each fatty acid was given as a relative percentage of the whole phospholipid trace. The values were then statistically analysed as described later in order to draw comparisons between the phospholipid fatty acid compositions of term, preterm and DNI neonates.

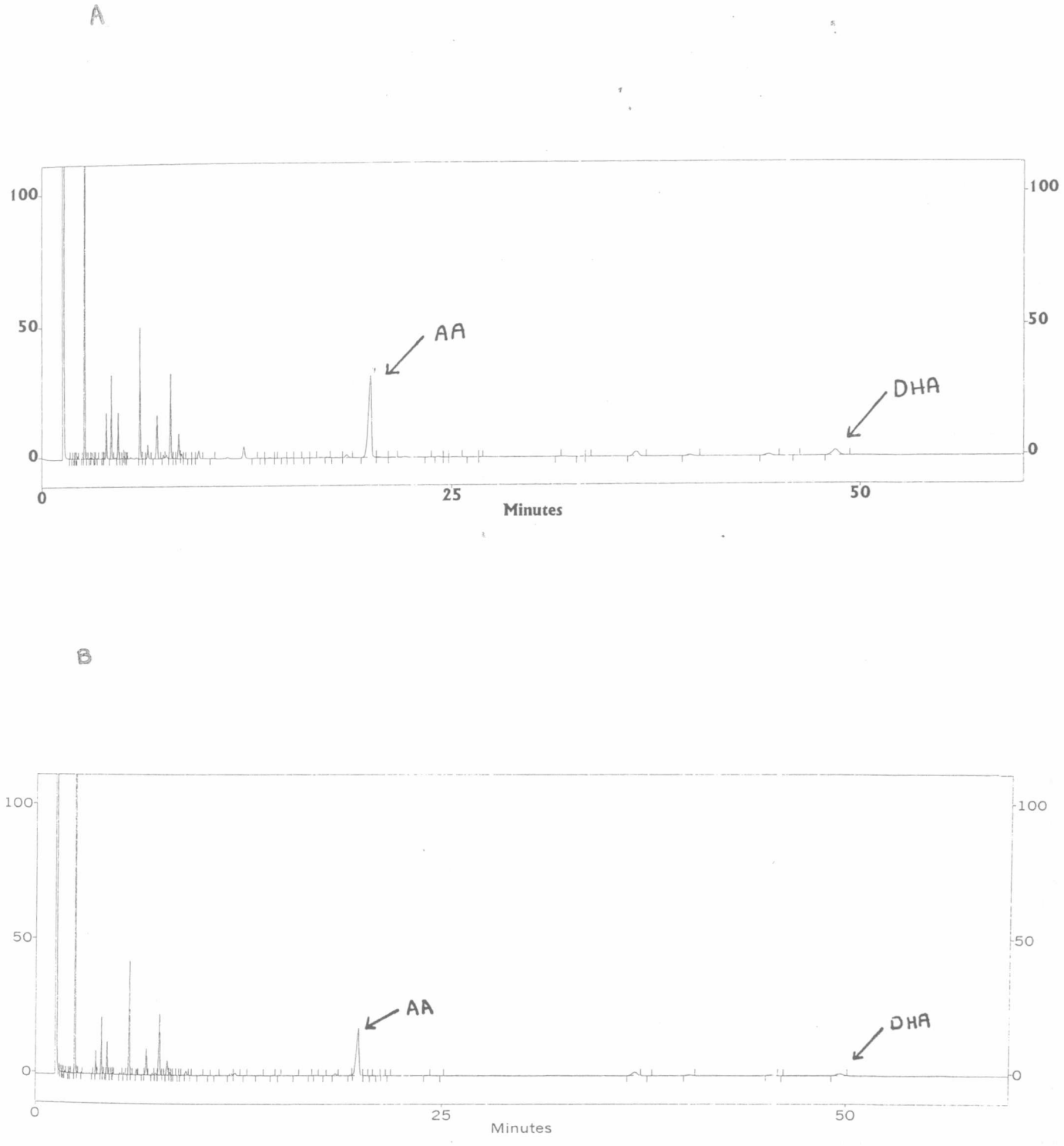


Figure 2.14: The gas chromatography histograms for a term (a) and preterm (b) neonate, clearly showing differences in the AA and DHA peaks.

2.5 Cell proliferation of CBMCs in the presence of AA and DHA

Experiments in cell culture and aseptic incubations are quite costly and require specialised equipment and many replicates of each sample which require large volumes of cord blood. In this study some of these requirements were lacking, and therefore limited the number and types of experiments that were carried out. In order to answer some of the fundamental questions about the effects of AA and DHA several simple though informative procedures were carried out as are recorded further on. The primary experiments included the use of colorimetry (MTS assay) as a means of assessing cell viability, and flow cytometric methods (as described above) to assess the proliferation of immunophenotype subsets. The combinations of the media and techniques used in the cell culture experiments are described in the following sections.

2.5.1 Cell culture conditions

All the isolation procedures for cord blood samples used for cell culture as well as the experiments were conducted under sterile conditions (section 2.3). The media and cells were prepared and plated under laminar flow (Heraeus Flow Cabinet). The plates were incubated for the respective culture times in a sterile cell culture incubator at 37°C and 5% CO_2.

As part of the experimentation was done on term cord blood taken from neonates born to HIV-positive women, special care was taken with the samples. At the stage of collection and processing for proliferation studies, the HIV status of the neonates was unknown. Safety precautions when working with potentially retrovirus infected samples orsdinarily require category 3 facilities, which unfortunately was not available. The experiments were therefore conducted under non-sterile conditions in a laboratory used for flow cytometric analysis of HIV-positive samples. The laboratory contained a 37°C

incubator, which did not have a CO_2 source. The decision was taken by the supervisory team that the cell culture should be conducted for a maximum of 4 hours under these conditions as soon as samples became available. Longer culture times would have exposed the cells to bacterial and fungal infections. Nevertheless sterile and non-touch techniques were maintained as far as possible.

2.5.2 Cell counting

CBMCs were extracted from whole umbilical cord blood as described previously,. However, for the cell proliferation studies the wash steps were carried out with RPMI 1640 (Roswell Park Memorial Institute medium – Sigma, appendix 1) which is routinely used in the culture of lymphocytes. Prior to plating the cells the cell number of the CBMC samples were counted using the Trypan Blue Dye Exclusion method. Trypan Blue is a negatively charged chromopore that reacts with cells with damaged membranes, meaning that cells that excluded the dye were viable (Freshney, 1983).

Briefly, 0.1ml of 0.4% Trypan Blue (Sigma) was added to 0.1ml of the cell suspension (after washing) and mixed thoroughly. This was allowed to stand for 5 minutes at room temperature (15-30^{o}C). The Trypan Blue dye was always filtered before hand to remove residue with a 0.45μ filter (Millipore). Thereafter a haemocytometer counter (figure 2.15) was filled with the mixture and the number of viable cells was counted under a light microscope. The count was determined using the following equation:

$$\textbf{Cells/ml} = n/v \times \text{dilution factor} \times 10^{6}$$

Where: n = number of cells counted

v = area (number of big squares counted) x depth (0.1)

Dilution factor = 2 (equal volumes of cell suspension and trypan blue)

Based on this the cell count of the original cell suspension was adjusted to 5 x 10^4 cells/100µl. Six replicates of each concentration or time study was conducted in order to reduce variability within the test samples. Original cell numbers of 1 x 10^5 cell/100µl were not possible due to the low numbers of cells obtainable from cord blood were insufficient to conduct the experiments in replicates of six.

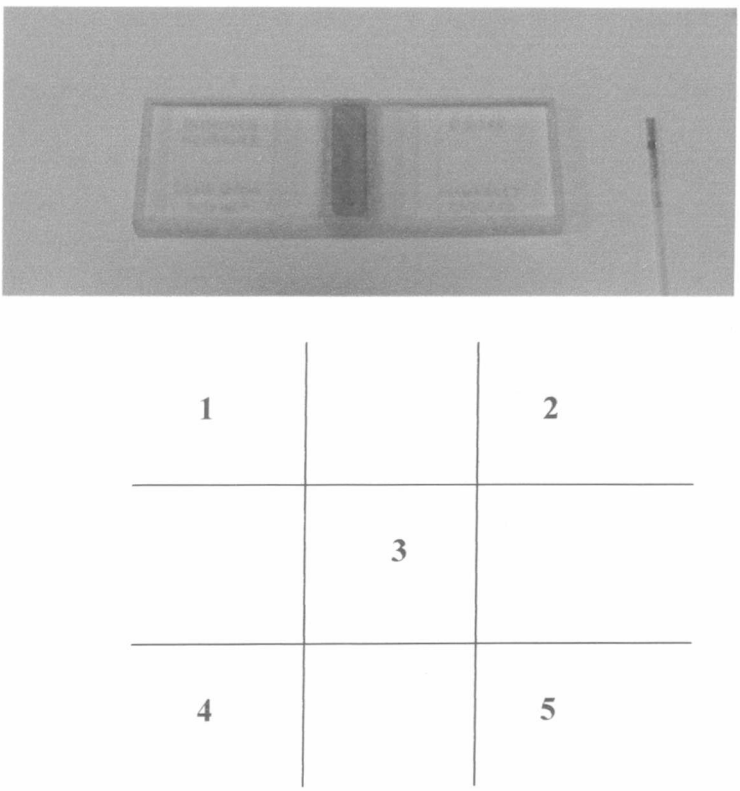


Figure 2.15: The Trypan Blue Dye Exclusion Assay. The chamber of the haemocytometer slide was filled with a mixture of the cll suspension and 0.4% trypan blue. Below the slide is a rudimentary sketch of the haemocytometer grid. Each of the 5 squares would contain a further 16 squares, apart from square 3, which contains 25 smaller squares.

2.5.3 Cell culture media

2.5.3.1 Standard culture medium

RPMI 1640 was used as the basic cell culture medium. To this 10% fetal calf serum (FCS-Gibco) and 1% penicillin and streptomycin (P/S - 5000units/ml penicillin and 5mg/ml streptomycin in 0.9% sodium chloride, Sigma) was added. The RPMI medium already contained L-glutamine (0.3g/L – appendix 1). This standard media was used in all the experiments and phytohaemagglutinin, AA and DHA were added for specific experiments. FCS does contain DHA although the amount is not known (pers comm. Dr. K. White, London Metropolitan University). The medium was warmed at 37°C before use.

2.5.3.2 Phytohaemagglutinin

Phytohaemagglutinin (PHA; Sigma) is a lectin isolated from *Phaseolus vulgaris* and is commonly used in the culture of lymphocytes. It is a mitogen which means that it stimulates cells to enter the S phase of the cell cycle, followed by G2 and mitosis (Becker *et al.*, 2005) thus enabling the proliferation of lymphocytes in culture. PHA is a T-cell mitogen (Zhao *et al.*, 2002).

PHA was added to the media of cells cultured for 72 hours to assess the function of term CBMCs i.e. to test the cell viability (MTS assay) and cell proliferation (flow cytometry) after 3 days in culture. PHA was added to the standard culture medium alone at 5μg/ml or together with concentrations of AA (32-256μM) in preliminary cell culture experiments with term CBMCs.

2.5.4 Concentrations of AA and DHA

AA and DHA were purchased as free fatty acids (Sigma). The ampoules were carefully broken in a darkened room. Dimethylsulfoxide (DMSO - 300µl) was added to the ampoules. DMSO can solubilise organic substances and was used with care as it has the ability of penetrating through rubber and skin and carrying its solubilised organic contents into circulation (Hay, 1992). The AA and DHA were then aliquoted into glass vials, flushed with N_2 gas and stored at -20°C in a darkened sealed container, to prevent peroxidation of the fatty acids.

Fresh CBMCs suspended in RPMI 1640 after washing were kept warm at 37°C, until the multiwell plates were prepared. The suspension was plated at 100µl (5 x 10^4 cells) per well which. Prior to experimentation the standard media were prepared and warmed at 37°C, and the workplace prepared before the stock concentrations of AA and DHA were solubilised and added to 10ml of standard medium to make stock solutions of 100µg/ml of AA and DHA. The concentrations of AA and DHA that were used were prepared by dilution of the stock solutions with standard medium and are shown in table 2.3. The cells were pelleted by centrifugation at 1000 RPM in a plate centrifuge. The supernatants were carefully discarded under laminar flow, and the medium containing AA or DHA were added to appropriate wells to a volume of 200µl in replicates of six. The plates were then incubated for designated periods of time at 37°C.

Table 2.3: The concentrations of AA and DHA used in the experiments expressed in micromolar (μM) of the fatty acids and μg/ml of standard medium.

μM AA	μM DHA	μg of AA or DHA per ml of standard medium
32	30	10
64	61	20
128	122	40
256	244	80
327	308	100
490	456	150
653	608	200

2.5.5 Cell viability using colorimetric analysis (MTS assay)

The most common method of assaying proliferated cells is by incubating them in media containing antigenic or mitogenic material for 3-7 days and thereafter including radiolabelled tritiated thymidine (^{3}H-Thy) into the DNA of dividing cells over 6-18 hours (Hickling, 1998). Some stimuli may induce immediate and rapid proliferation and become toxic over longer incubation periods (Hickling, 1998). The use of MTT* and MTS assays are also widely used to assess the cell viability and proliferation of cells in culture.

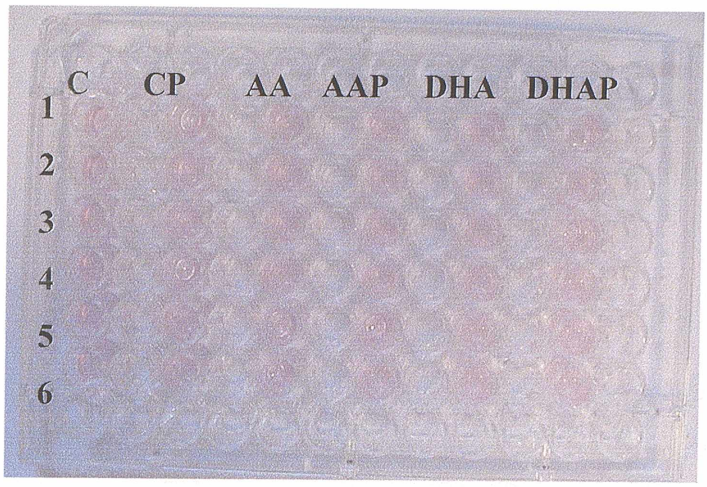
* MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

The method may include cells that have been cultured with a mitogen or antigenic stimulus and either an untreated batch of cells (referred to as the control) of a batch of cells treated with a specific antigen to determine the specificity of the response.

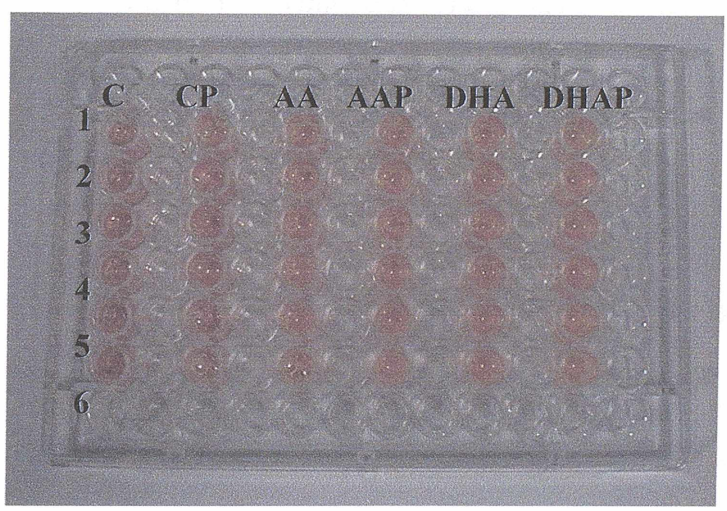
The MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] is a one-solution non-radioactive product and contains phenazine methosulfate (PMS) which is bioreduced by living cells to produce a formazan product that is readily soluble in the medium (Buttke *et al.*, 1993). The MTS (Promega) used in this thesis contained PES (phenazine ethosulfate), which is a electron coupling reagent that is combined with MTS to form a stable solution (Promega protocol, 2001). MTS has the advantage over the MTT reagent in that it does not require solubilization by products such as DMSO (Reubel *et al.*, 1997). The use of the MTS reagent has been advocated as a microculture tetrazolium assay as well as to screen the effects of large numbers of drugs at wide concentration ranges (Goodwin *et al.*, 1995 and Cory *et al.*, 1991).

After the specified incubation time, 20µl of MTS reagent per 100µl of cell culture medium was added to each well, and the plates were reincubated for a further 1 hour at 37°C. The absorbance was read on a microplate reader (Anthos) at 492nm (reference wavelength 620nm). Figure 2.16A shows a plate with cells cultured with standard media containing fatty acids with and without PHA. Figure 2.16B shows the same plate after MTS reagent was added, and figure 2.16C shows that the deeper colour of formazan produced by living cells was seen in plates incubated with MTS for 1 hour.

A



B



C

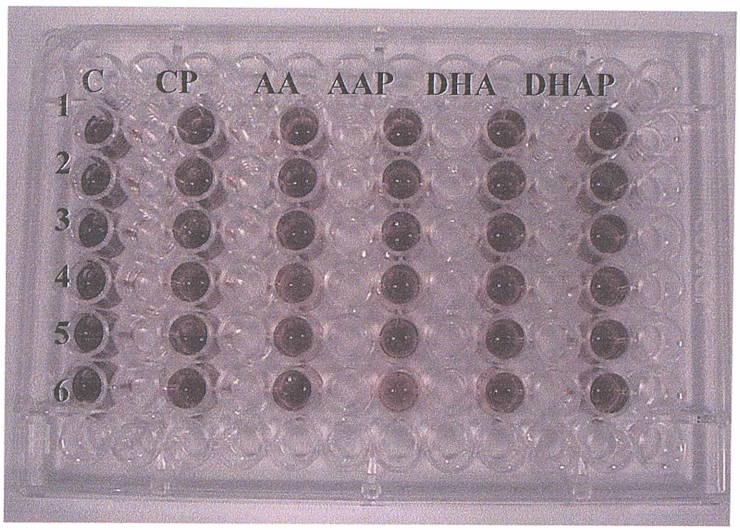


Figure 2.16: The incubation of cultured cells with MTS reagent, where A shows the light pink colour of the media containing CBMCs, B shows the colour of the wells after MTS is added and C shows the colour of the resultant formazan product after phenazine methosulfate has been reduced by living cells. These plates are also representative of how the HT and DNI experiment were set up. Another set of three identical plates were prepared for the analysis of immunological subsets.

2.5.6 The effect of AA and DHA supplementation on the proliferation of lymphocytes

Preliminary experiments were carried out over conducted over 72 hours in order to assess the proliferative response of term CBMCs to PHA and AA. These experiments involved the analysis of immune cell subsets by flow cytometry. The cells were plated as described previously. The media was removed afyter plate centrifugation and culture media containing PHA alone, or PHA with AA (256μM) were added to the approporiate wells. The flow cytometric analysis was carried out in exactly the same way as described in section 2.2.2. The immune subsets analysed are outlined in table 2.2.

2.6 Data analysis

Most of the results are expressed as mean and standard deviation values. The statistical significance of the difference between the absolute cell number obtained for the immune subset counts of term, preterm and DNI samples (chapter 3) as well as the relative percentage membrane fatty acid composition of term, preterm and DNI samples (chapter 4) were analysed using the Kruskal-Wallis method for non-parametric data (StatsDirect, statistical software 2006, version 2.5.5) and expressed using the Dwass-Steel-Chritchlow-Fligner all pairwise comparison.

The relationship between gestational age and absolute cell count using the term and preterm data (chapter 3) was given by a correlation coefficient (r) using parametric methods for normally distributed data. Significance of correlation was calculated using the t-test based on standard deviation between r and zero (Kirkwood, 1988) and confirmed using StatsDirect, statistical software 2006, version 2.5.5.

The correlation between AA and birthweight and between DHA and gestational age (chapter 3) as well as the correlation between fatty acid percentages and immune subset levels (chapter 5) was given by Spearman's rank correlation for non-parametric measures (r). Significance was calculated using the t-test based on standard deviation between r and zero using StatsDirect, statistical software 2006, version 2.5.5.

Chapter 3

Cord blood mononuclear cell characterisation by immune flow cytometry

3.1 Introduction

Preterm and DNI neonates are at a distinct disadvantage immunologically as they are not fully immunocompetent at the time of birth. This has mainly been attributed to differences in the numbers and function of immune cells at birth between healthy term neonates and preterm and DNI neonates.

Preterm neonates are more susceptible to infection than term neonates (Lilja *et al.*, 1984) and may have resultant complications with respiration and cerebral haemorrhaging. These babies are born at a time when they have not received the full complement of placental nourishment (Crawford *et al.*, 1998), but due to their small gestational ages are also subjected to lower numbers of T-lymphocytes compared to neonates born at term (Juretić *et al.*, 2000). Preterm neonates were reported to have lower percentages of CD3 T-cells, particularly T-helper (CD4) cells (Juretić *et al.*, 2000), and the cell numbers were reported to be lower in preterm neonates presenting with early perinatal bacterial infection compared to uninfected preterms (Juretić *et al.*, 2001).

Babies born to HIV-positive women are at risk of vertical transmission (mother to child transfer). However it has emerged in recent years that even the uninfected neonates of HIV-positive women (DNI) have certain immunological deficiencies when compared to healthy neonates of uninfected women. DNI neonates have been reported to have low T-cell counts, low CD4:CD8 ratios, poor T-cell function as well as a higher number of activated and memory cells (Nielson *et al.*, 2001, Rich *et al.*, 1997 and Clerici *et al.*, 2000). Although, antiretroviral drug therapy, caesarean section deliveries and

prevention of breast-feeding have reduced the risk of mother-to-child transfer of the virus appreciably, these measures pose risks of their own in compromising the immune system function of the newborn.

The aim of the following work was to establish a comparison of the immunophenotype subset levels between term, preterm and DNI cord blood lymphocytes, in order to establish a basic understanding of immune system maturation at the time of birth.

3.2. Methods

3.2.1. Subjects and sample collection: Women delivering at term (37-40+ weeks) and preterm (30-35 weeks) were recruited at Newham University Hospital, London prior to delivery and informed consent obtained for the collection of cord blood samples. Women were aged between 20-40 years with a parity of 0-3. Prior to delivery women in preterm labour were treated with dexamethasone sodium phosphate (dexamethasone, 12mg); a majority of women (n=14) received a second dose approximately 12 hours after the first dose. However 3 women did not receive dexamethasone treatment at all.

All HIV-positive women were delivered by caesarean section and in addition to their own antiretroviral drugs, zidovudine (AZT) was routinely administered intravenously throughout surgery until the umbilical cord was clamped and cut. At birth, all the participating neonates were healthy in that they were not suffering nosocomial infection nor infection acquired *in utero* or inflammatory reactions.

All the neonates of HIV-positive women that participated in this study were determined to be HIV negative at 6 months after birth, and are referred to here as definitely not infected (DNI) in accordance with Centre for Disease Control (CDC)

recommendations[5]. Data relating to the mothers and their babies is shown on table 2.1. Consequently the immune status of the term (n=23), preterm (n=24) and DNI (n=9) neonates that participated in this study were compared by measuring the statistical difference between the means of relevant immunophenotypes (CD markers). In addition the levels of these markers in term and preterm cord blood were correlated with gestational age.

3.2.2. Flow Cytometry Analysis

Lymphocyte subsets present in fresh cord blood samples were analysed by four-colour flow cytometry using a Q-Prep automated system (Beckman Coulter) and an EPICS XL-4 flow cytometer (Beckman Coulter). The fluorochrome-conjugated monoclonal antibodies (IOtest; Beckman Coulter) raised against lymphocyte cluster of differentiation (CD) antigens used were: T-cell, FITC-CD4 (clone 13B8.2); ECD-CD3 (clone UCHT1); PC5-CD8 (cloneB9.11); memory/naïve, PE-CD45/RA (clone ALB11); ECD-CD45RO (clone UCHL1); B-cell, FITC-CD19 (clone J4.119); pan-leukocyte, PE-CD45 (clone J33); and activated (IL-2-expressing), PC5-CD25. Appropriate isotype controls for IgG1 Abs (all FITC-,PE-and-ECD-conjugates) and IgG2a (PC5-conjugates were clones 79.1Mc7 and U7.27 respectively). Prior to analysis, 100µl of whole cord blood was incubated with combinations of monoclonal antibodies (described above) for 10 minutes. Red blood cells were lysed, white blood cells fixed (ImmunoPrep Reagent System, Beckman Coulter) and absolute counts (cells/µl) for each phenotype obtained.

3.2.3. Data analysis:

Results are expressed as mean and standard error. The significance of the difference between the absolute cell number obtained for the CBMC subset counts of term, preterm and DNI samples were analysed using the Kruskal-Wallis method for non-

parametric data (StatsDirect, statistical software 2006, version 2.5.5) and expressed using the Dwass-Steel-Chritchlow-Fligner all pairwise comparison. The relationship between gestational age and absolute cell count using the term and preterm data was given by a correlation coefficient (r). The correlation coefficient for the term and preterm data was analysed using parametric methods for normally distributed data. The normal distribution of term and preterm data is demonstrated in figure 3.1, where the upper and lower quartiles are fairly similar, while there is an obvious skew in the DNI data. Significance was calculated using the t-test based on standard deviation between r and zero (Kirkwood, 1988) and confirmed using StatsDirect, statistical software 2006, version 2.5.5.

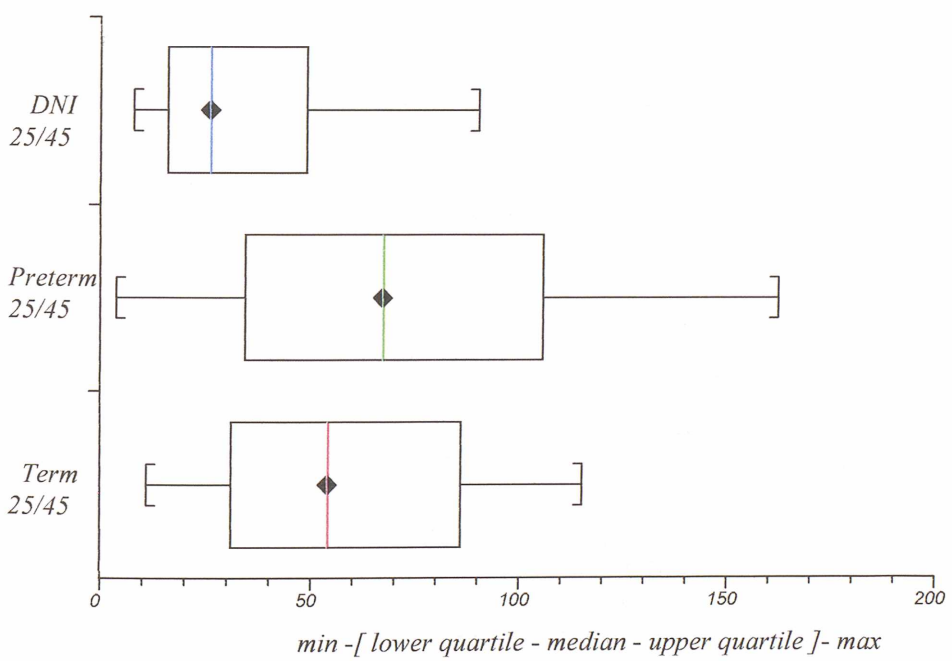


Figure 3.1. Box and Whisker plot of CD25/45 counts between term, preterm and DNI CBMCs (StatsDirect, version 2.5.5)

3.3. Results

Table 3.1: Cell counts of term, preterm and DNI cord blood mononuclear cells.

CD Antigens	Mean ± SE cells/μl (Median and interquartile range)			Kruskal-Wallis test for significance (p value)		
	Term (n=23)	Preterm (n=24)	DNI (n=9)	Term & Preterm	Term & DNI	Preterm & DNI
CD4/45	1826 ±136.01 (1941: 1430-2041)	1428 ±103.16 (1382: 1132-1737)	1342 ±120.25 (1317: 1224-1562)	<0.05	<0.05	NS
CD8/45	946 ±76.04 (957:727-1162	730 ±47.69 (641:600-861)	849 ±160.30 (814:442-1050)	NS	NS	NS
CD3/4	1668 ±114.82 (1788:1099-1897)	1406 ±102.37 (1369:1106-1696)	1326 ±117.49 (1312:1219-1534)	NS	NS	NS
CD3/45	2377 ±146.41 (2485:1743-2812)	1995 ±135.37 (1940:1597-2342)	2097 ±267.76 (1939:1564-2423)	NS	NS	NS
CD3/8	630 ±47.84 (602:432-828)	555 ±44.83 (497:426-668)	703 ±153.65 (556:348-727)	NS	NS	NS
CD25/45	57 ± 6.80 (54:31-86)	69 ± 8.09 (67:35-101)	34 ± 8.69 (26:20-38)	NS	NS	<0.05
CD19/45	616 ±72.50 (565:328-982)	719 ±102.21 (572:467-963)	567 ±120.37 (394:316-756)	NS	NS	NS
CD3/25	35 ± 5.10 (31:10-56)	52 ± 5.95 (54:29-67)	19 ± 5.86 (13: 9-18)	NS	NS	<0.01
CD8/RA	1034 ±77.92 (965:742-1220)	739 ± 64 (788:570-900)	807 ± 140.16 (840:392-989)	<0.05	NS	NS
CD4/RA	1546± 101.55 (1608:1312-1888)	1038± 102.77 (1108:725-1410)	885± 97.31 (845:701-1041)	<0.01	<0.01	NS
CD4/RO	651 ± 50.42 (628:530-711)	583 ± 44.58 (595:424-748)	421 ± 55.83 (421:350-508)	NS	<0.05	NS
CD8/RO	202 ±16.90 (182:144-225)	159 ± 18.61 (164:112-222)	123 ± 28.93 (107:63-165)	NS	NS	NS

Mean absolute counts from term, preterm and DNI, where: CD4 (T-helper cells); CD3 (Pan T-cells); CD8 (cytotoxic T-cells); CD25 (activated/ interleukin-2 responsive cells); CD45 (Pan leucocytes); CD19 (B-cells); CD45RA (naïve cells); CD45RO (memory cells) and *NS is no significant difference. Significant difference between the means is shown by $p<0.05$.

Table 3.1 describes the mean absolute cell count per microlitre of each immunophenotype for term, preterm and DNI neonates. In addition the standard error, median and interquartile range for each group is included. Statistical difference (p value) between the means is given by the Kruskal-Wallis test (StatsDirect, version 2.5.5).

CD3/45 lymphocytes express the T-cell (CD3) and leukocyte (CD45) antigens. These CD antigens are therefore carried by all T-lymphocytes, and give an account of the total T-lymphocyte count in a sample. Table 3.1 shows that preterm and DNI cord blood contained smaller numbers of T-lymphocytes than term neonatal cord blood. Preterm cord blood contained the lowest levels of CD3/45 lymphocytes.

CD4 is specific to T-helper cells within a population of leukocytes. Table 3.1 shows that the number of T-helper cells in the leukocyte population (CD4/45) was significantly lower in preterm and DNI cord blood compared to term ($p<0.05$). CD4/45 levels in DNI neonates were the lowest of the 3 groups. When the T-helper cells within the T-cell population was assessed alone (CD3/4), preterm and DNI neonates had lower levels of these lymphocytes than term neonates, but there was no statistical significance in the difference between the groups. DNI cord blood contained the lowest levels of CD3/4 lymphocytes than term or preterm cord blood.

Naïve T-lymphocytes (CD4/45RA) were significantly higher in term cord blood ($p<0.01$) than in either preterm or DNI. DNI levels of CD4/45RA were lower than preterm levels, though not significantly different. Term neonates had higher numbers of memory CD4 cells (CD4/45RO) than preterm (not significant) or DNI neonates ($p<0.05$).

Preterm cord blood had the lowest cytotoxic leukocyte (CD8/45) levels of the three groups however there was no significant difference between the groups (table 3.1). The counts for the DNI neonates were less than term, but closer to term than preterm values. However DNI cord blood had higher levels of cytotoxic T-cells (CD3/8) than term or preterm cord blood (not significant).Term cord blood contained higher levels of CD8 naïve cells (CD8/45RA) than preterm ($p<0.05$) and DNI (not significant). Both preterm and DNI cord blood contained smaller counts of CD8/RO (memory) cells compared to term cord blood. There was no significant difference between the groups.

Preterm cord blood contained higher numbers of interleukin-2 (IL-2) responsive leukocytes (CD25/45) than term (no significance) or DNI ($p<0.05$) cord blood. More specifically preterm cord blood contained higher numbers of IL-2 responsive T-cells (CD3/25) than term or DNI ($p<0.01$) cord blood (table 3.1). Preterm cord blood also contained higher levels of B-cells (CD19/45) than term or DNI neonates, although there was no significant difference between the groups.

Table 3.2: The correlation between immunophenotype and gestational age in days.

CD antigens	Correlation coefficient (r)	p value
CD3/45	0.4	<0.01
CD4/45	0.4	<0.01
CD8/45	0.4	<0.01
CD3/4	0.3	<0.05
CD3/8	0.3	<0.05
CD3/25	-0.3	<0.05
CD25/45	-0.2	NS
CD8/RA	0.3	<0.05
CD4/RA	0.3	<0.05
CD4/RO	0.1	NS
CD8/RO	0.1	NS

Correlation between T-cell subsets and gestational age determined from the absolute counts determined for 47 individual neonates from 100µl of whole cord blood incubated with fluorochrome-conjugated monoclonal antibodies raised against the lymphocyte cluster of differentiation (CD) antigens shown, where: CD4 (T-helper cells); CD3 (Pan T-cells); CD8 (cytotoxic T-cells); CD25 (activated/ interleukin-2 responsive cells); CD45 (Pan leucocytes); CD45RA (naïve cells); CD45RO (memory cells); r = correlation coefficient (appendix); NS is no significant difference; p value <0.05 was significant.

Table 3.2 shows that there was significant positive correlation between the numbers of leukocytes (CD3/45, CD4/45 and CD8/45) and T-lymphocytes (CD3/4 and CD3/8) with gestational age ($p<0.01$-0.05). Positive correlations were also shown between gestational age and CD4 and CD8 naïve cells ($p<0.05$). However, there was a negative correlation between IL-2-responsive cells, particularly between CD3/25 ($p<0.05$) and gestational age at birth.

Women delivering preterm at Newham General Hospital were routinely given dexamethasone to mature the fetus's lungs in time for delivery. As dexamethasone has been implicated in decreased T-cell counts in preterm neonates (Chabra *et al.*, 1998), it was of importance to determine if the difference in term and preterm immunophenotypes seen above (table 3.1) resulted from the effect of the drugs, or was truly associated with gestational age. As term neonates are not exposed to dexamethasone this was difficult to do. Therefore for the purposes of this study the cell counts of T-leukocyte phenotypes were compared between dexamethasone exposed and unexposed neonates (table 3.3) as well as between preterm neonates exposed to either 1 or 2 doses of dexamethasone.

Table 3.3: The comparison of leukocyte counts in the cord blood of preterm neonates exposed to dexamethasone compared to unexposed preterm neonates.

	Mean ± SE cells/μl (Median and interquartile range)		**Kruskal-Wallis test for significance (p value)**
	Dexamethasone exposed (n=21)	**Dexamethasone unexposed (n=3)**	
CD45	3210 ± 174 2979: 2574 - 3406	2752 ± 741 3370: 1275 - 3612	NS
CD4/45	1444 ± 107 1359:1185 - 1722	1318 ± 409 1525: 529 - 1901	NS
CD8/45	740 ± 50 637: 600 - 842	661 ± 179 743: 318 - 921	NS
CD3/4	1423 ± 107 1352: 1162 - 1665	1287 ± 394 1503: 522 - 1835	NS

Mean absolute counts from preterm neonates exposed to dexamethasone *in utero*, where: NS is not significant; CD3 (Pan T-cells); CD4 (T-helper cells); CD8 (cytotoxic T-cells) and CD45 (Pan leucocytes).

No significant difference was found between the CBMC immunophenotypes between preterm neonates exposed to dexamethasone compared to neonates from untreated women (table 3.3). The unexposed preterm samples contained fewer immune cells than the dexamethasone treated neonates.

Table 3.4: The comparison of leukocyte counts in the cord blood of preterm neonates exposed to 1 dose dexamethasone compared to those exposed to 2 doses.

	Mean ± SE cells/μl (Median and interquartile range)		**Kruskal-Wallis test for significance (p value)**
	1 dose exposure to dexamethasone	**2 dose exposure to dexamethasone**	
CD45	2817 ± 142 2932: 2536 - 3207	3407 ± 238 3126: 2833 - 3826	NS
CD4/45	1165 ± 155 1006: 761 - 1437	1583 ± 129 1424: 1218 – 1751	NS
CD8/45	757 ± 81 637: 600 - 946	731 ± 65 638: 600 - 824	NS
CD3/4	1150 ± 157 990: 742 - 1425	1559 ± 128 1404: 1198 - 1728	NS

Mean absolute counts from preterm neonates exposed to 1 or 2 doses of dexamethasone *in utero*, where: NS is not significant; CD3 (Pan T-cells); CD4 (T-helper cells); CD8 (cytotoxic T-cells) and CD45 (Pan leucocytes).

There was no significant difference in the numbers of T-leukocytes between preterm neonates exposed to 1 dose or 2 doses of dexamethasone prior to delivery. Although not significantly different, neonates exposed to 2 doses of dexamethasone had relatively comparative and sometimes higher cells numbers than those exposed to 1 dose (table 3.4).

3.4 Discussion

It is known that preterm and DNI neonates are at a distinct immune disadvantage when compared to healthy neonates born at term (Juretić *et al.*,2001 and Clerici *et al.*, 2000). A common way of assessing immune competence is to analyse the absolute cell number of immune cell subsets by immunolablelling and flow cytometric methods. The immune system of a healthy neonate is given by an increase in T-cells from birth to later infancy, particularly with regards to CD4/45RA, CD3/4 and CD3/8 positively labelled lymphocytes (Raes *et al.*, 1993 and Stern *et al.*, 1992). These CD antigens correspond to specific functions that facilitate the optimum functioning of the immune cells.

The absolute cell number for the various T-cell subsets that were observed for the term neonates in this study were in agreement with the findings of other workers (De Vries *et al.*, 2000, Comans-Bitter *et al.*, 1997 and Kontny *et al.*, 1994). The subset values for term cord blood were therefore considered a suitable standard against which cell subset levels from preterm and DNI cord blood could be compared.

In comparison with term neonates, the immune system of preterm neonates is less competent (Juretić *et al.*, 2001). It is not clear whether this is related to decreased numbers of immune cells or increased numbers of undifferentiated or unlabelled cells (Juretić *et al.*, 2000 and Juretić *et al.*, 2001). Preterm neonates are susceptible to inflammation due to mechanical ventilation procedures as a result of small and poorly formed lungs, which eventually results in BPD (Jobe and Ikegami, 1998). The reasons for persistent inflammation are poorly understood. Duggan *et al.*, 2001, showed that very preterm neonates (<30 weeks gestation) who were infected *in utero* presented with inflammatory brain lesions; and these workers were able correlate the lesions with the presence of inflammatory cytokines and CD45RO memory cells.

Table 3.1 shows that preterm neonates compared to term neonates had lower levels of CD3/45 (not significant), CD4/45 ($p<0.05$), CD3/4 (not significant), CD4/45RA ($p<0.01$), CD4/45RO (not significant), CD8/45 (not significant), CD3/8 (not significant), CD8/45RA ($p<0.05$) and CD8/45RO (not significant). This data was similar to work done by Bussel *et al.*, 1988, where preterm neonates were shown to have lower percentages of CD3 and CD8 cells at birth when compared to term neonates. The low levels of CD4 and CD8 lymphocytes in the preterm cord blood compared to term, suggests that there was a degree of immaturity in the preterm immune system,

relevant to the stage of development at that time of birth. Importantly, this study showed significant positive correlation between gestational age and leukocytes, T-lymphocytes and CD4 and CD8 naïve cells (table 3.2). The lower number of CD4 and CD8 memory cells (CD45RO) as opposed to findings of high levels of these cells and inflammatory cytokines by Duggan *et al.*, 2001, implies the absence of fetal infection.

Table 3.1 shows that preterm cord blood had a higher mean level of IL-2 responsive T-cells (CD3/25; not significant). The synthesis and secretion of IL-2 is triggered by antigen or mitogen-stimulated activation of mature T-lymphocytes and signals the differentiation, proliferation and maturation of a variety of haematopoietic cells (Goldsmith and Greene, 1994). Table 3.2 also shows that there was a significant negative correlation between the CD3/25 lymphocytes and gestational age at birth ($p<0.05$). This data indicates that preterm neonates have higher numbers of T-cells that are responsive to IL-2 and may correspond to a time when proliferation and maturation are about to occur. The low levels of memory cells (table 3.1) seems to indicate that the high levels of CD25+ T-cells in preterm cord blood may not be as a response to antigen activation, but rather as a response to proliferation and maturation as a normal developmental process.

The results from the current study have shown that there was also a positive correlation between the naïve CD45RA cells and gestational age at birth. Early work by Meister *et al.* (1994) indicated that preterm umbilical cord blood contained up to twice the number of CD34+ (haematopoietic precursor cells or stem cells) than term cord blood. In that study, a significant ($p<0.001$) number of the CD34+ cells were found to express CD71+, which is a marker on cells committed to erythroid rather than myeloid lineage. Those workers also showed a significant positive correlation between the

myeloid/erythroid ratio and gestational age. This may account for the lower numbers of CD4 and CD8 T-cells and CD4 and CD8 naïve cells reported here in preterm cord blood. Thus lower number of T-cells in preterm neonates may be a consequence of the higher cell number committed to the erythroid lineage.

The influence of antenatal steroids on the immune subset levels was also considered. The use of antenatal steroids in perinatal care, was first introduced by Liggins and Howie in the 1970s when they showed that these drugs reduced the risk of RDS in preterm neonates (Liggins and Howie, 1972). By the 1990s dexamethasone and betamethasone were recommended for antenatal therapy[6].

The majority of mothers of preterm babies in this study received 2 doses of 12mg, dexamethasone prior to delivery. Dexamethasone and betamethasone have the same biologic activity and do cross the placenta (Klinger and Koren, 2000). Chabra *et al.* (1998), attributed a decrease in CD4 and CD8 T-cells with decreasing gestational age to the use of antenatal steroids. They found that CBMCs of neonates born to women who were administered antenatal steroids had significantly lower absolute lymphocyte counts ($p<0.0001$), as well as lower CD4 ($p<0.02$) and lower CD25 ($p<0.03$) T-cells. Although these findings are similar to some of the data presented here, absolute counts for the CD25 population in the preterm babies that participated in this study were higher than that of term CBMCs (table 3.1) albeit not significantly. In addition, it was unclear from the Chabra *et al.* (1998) report which class of antenatal steroid was used, nor the concentration used or the number of doses given to the women delivering preterm.

Other reports give conflicting results; Kotiranta-Ainamo *et al.*, (1999) looked at the effects of antenatal steroids (betamethasone in particular) on the immune cells of

preterm neonates. These workers also considered the independent effects of gestational age, neonatal infection, maternal pre-eclampsia and mode of delivery and concluded that betamethasone therapy increased proportions of T-cells and macrophages but reduced the proportion of natural killer cells, and therefore did not pose any significant immunosuppressive risk. Two other groups reported that both dexamethasone and betamethasone have a relatively weak immunosuppressive effect on peripheral blood lymphocyte function (Klinger and Koren, 2000 and Ryhanen *et al.*, 1980), yet others consider them to have a more potent effect (Parimi *et al.*, 1999 and Schwarze and Bartmann, 1994). However these latter workers' evaluation of dexamethasone was based on postnatal treatment of preterm neonates for BPD, which required a prolonged course of medication for up to six weeks (Schwarze and Bartmann, 1994) when immunosuppressive effects would be expected.

Preterm neonates (21 of 24) were born to mothers who received either one or two doses of dexamethasone at least 12 hours prior to delivery, whilst 3 of 24 babies were unexposed. The lymphocyte subset counts in the dexamethasone-treated group did not differ significantly from the untreated group (table 3.3). Furthermore there were no significant differences in the lymphocyte subset counts between neonates exposed to 1 or 2 doses of dexamethasone in utero (table 3.4). In fact the group treated with 2 doses seemed to have higher rather than lower levels of CD4 leukocytes and lymphocytes. Consequently it was considered highly unlikely that the antenatal use of dexamethasone accounted for the results presented in this thesis.

The use of antiretroviral drugs together with caesarean section delivery and prevention of breast-feeding in HIV-positive women has lowered the risk of vertical transmission of the virus to the neonate. However there is some evidence to show that HIV-negative

neonates born to HIV-positive mothers may not be as immunocompetent as uninfected term neonates (Clerici *et al.*, 2000). Table 3.1 shows that compared to term levels, DNI CBMCs contained lower levels of T-cells in the leukocyte population (CD3/45; not significant), CD4 T-cells (not significant) and CD4 leukocytes ($p<0.05$). The DNI cord blood contained slightly higher levels of these subsets compared to preterm cord blood, although none of the differences were significant (table 3.1). Lower numbers of CD4+ (helper T-cells) as well as red blood cells have been reported by others and it has been suggested that this is due to the impairment in the function of progenitor cells (Nielson *et al.*, 2001). Others have shown that DNI cord blood T-lymphocytes are more susceptible to apoptosis (Economides *et al.*, 1998) which would therefore reduce their levels in circulation.

The cord blood of healthy uninfected term neonates born to uninfected women contained higher levels of CD4 naïve ($p<0.01$) and memory cells ($p<0.05$) compared to DNI cord blood. These results are not in complete agreement with Clerici *et al.*, 2000. Although these workers reported that the percentage of CD4 naïve cells was significantly higher in healthy term neonates as was shown in this study, they found that the CD4 memory cell level (CD4/45RO) was significantly higher in DNI neonates rather than in healthy uninfected neonates born to uninfected women.

Relative to term, DNI cord blood contained similar levels of CD8/45, CD3/8 and CD8/45RA lymphocytes (table 3.1), thereby decreasing the CD4:CD8 ratio. A lower CD4-expressing T-cell count with a consequently lower CD4:CD8 ratio in seroreverters was reported by Clerici *et al.* (2000). One explanation for high DNI cord blood levels of CD4/45RO and low CD4 T-cell levels reported by Clerici *et al.*, 2000, is the possible exposure of the foetus to HIV and/or to soluble and other viral proteins in the uterus of

the HIV-positive mother. Since HIV targets the CD4 receptor, impairment of CD4-expressing T-lymphocyte development or function may occur even in the HIV-uninfected (DNI) neonate (Nielson *et al.*, 2001 and Clerici *et al.*, 2000). It has been shown that the frequency of HIV-specific $CD4^+$ T-cells was augmented in DNI neonates, but that HIV-specific interferon-gamma secreting CD8 T-cells were present in only a small number of individuals, implying that intrauterine exposure of DNI neonates may have been subsequent to transplacental diffusion of HIV soluble proteins and not to replication competent viruses (Nielson *et al.*, 2001).

The paucity of CD4/45RO (memory) cells in the DNI cord blood in this study may imply that the fetuses were not subjected to viral particles or proteins, or perhaps that the maternal viral load in each case was too small have any significant impact on the CD4 memory cells of the neonates. However, conclusive evidence of this would have had to be tested by determining T-cell function by exposure of CD4 lymphocytes from these neonates to HIV viral coat proteins as mentioned in Clerici *et al.*, 2000 and Nielson *et al.*, 2001.

DNI cord blood expressed the lowest levels of CD25/45 and CD3/25 compared to term (not significant) and preterm ($p<0.05$ and $p<0.01$ respectively). This suggests that the DNI lymphocytes were not expressing IL-2 receptors. Previous literature has indicated that altered T-lymphocyte function demonstrated in DNI neonates may have result from the inability of T-cells to express the interleukin-2 ($CD25^+$) receptor (Rich *et al.*, 1997) or due to defects in the $CD4^+$ and $CD8^+$ (cytotoxic) subpopulations of T-cells (Clerici *et al.*, 2000).

Finally, antiretroviral treatment using Zidovudine (AZT), common during pregnancy as well as immediately before and during parturition, crosses the placenta and is a category C drug. Antiretrovirals have been implicated in perinatal genotoxicity, carcinogenicity and mitochondrial toxicity (Poirier *et al.*, 2003 and Poirier *et al.*, 2004), low birth weight (Kind *et al.*, 1998) and inhibition of bone marrow functions (Richman *et al.*, 1987). Conversely other studies do not show any serious adverse outcome to pregnancy (ECS, 2003 and Kind *et al.*,1998). Nevertheless, immune function may be affected in the DNIs but it was not possible to control for this since clearly HIV-infected women must be treated to reduce transmission to her baby and of course the mothers of HT neonates would not receive ARVs.

3.5 Conclusion

The aim of this study was to assess the immunophenotype subset levels in term, preterm and DNI CBMCs. The data revealed that preterm neonates had lower levels of T-cells specifically CD4/45, CD4/45RA and CD8/45RA compared to term neonates. DNI neonates had lower levels of CD4/45, CD4/45RA and CD4/45RO than term neonates. Preterm neonates had higher levels of CD3/25 and CD25/45 than term and DNI neonates. CD3/25 was also found to be significantly negatively correlated with gestational age. Statistical analyses showed that lower levels of preterm immune subsets were not as a result of exposure to antenatal dexamethasone treatment.

The results presented in this study are in agreement with others and have established that there are differences in immune system maturation in preterm and DNI neonates compared to healthy term neonates in regard to immunophenotype subset levels. These differences may be an indication of susceptibility of preterm neonates to chronic

inflammation and DNI neonates to poor immune competence and activation of helper T-cells.

As disruptions in LCPUFA supply may occur due to low gestational age or high maternal LCPUFA turnover rates in preterm and DNI births respectively, it is of importance to determine the level of fatty acids within CBMC membranes as a step towards determining the association between placental provision of fatty acids and the functioning of the immune cells.

Chapter 4

Cord blood mononuclear cell fatty acid composition by gas chromatography

4.1. Introduction

Long chain polyunsaturated fatty acids such as arachidonic acid (AA 20:4n-6) and docosahexaenoic acid (DHA 22:6n-3) are important components of cell membrane phospholipids. As reviewed in chapter 1, LCPUFAs serve as substrates for the synthesis of several biologically active eicosanoids and mediators of inflammatory resolution.

A positive correlation has been recorded between low cord blood plasma AA and low birth weight as well as between low cord blood DHA levels and gestational age (Leaf *et al.*, 1992). As gestation progresses the placenta selectively extracts AA and DHA from the mother and enriches the foetal circulation in particular the supply of AA is more than doubled (Crawford, 2000 and Crawford *et al.*, 2003). Thus, preterm neonates are born at a nutritionally disadvantageous time, whereas DNI neonates may be subjected to maternal wasting, or poor diet due to depression and malabsorption (Hellerstein et al., 1996) or high maternal LCPUFA turnover due to the disease.

Previous data have shown that preterm and DNI neonates are also at a higher immunological risk than term neonates (Juretić *et al.*, 2001 and Clerici *et al.*, 2000). The lipid profile of preterm and DNI CBMCs had not previously been examined, and it was therefore the intent of this study to attempt to establish the fatty acid composition of term, preterm and DNI CBMCs as a preliminary step in understanding the impact of deficits in vital LCPUFAs on the immune system of neonates.

4.2. Methods

4.2.1 Isolation of mononuclear cells from whole cord blood

Blood was taken under vacuum into EDTA from the umbilical vein and CBMCs isolated using standard techniques. Briefly, whole blood was layered onto Histopaque 1077 (Sigma) and centrifuged at 400xg for 30 minutes. The buffy coat was relayered on Histopaque 1077 as before to reduce erythrocyte and nucleated erythroid precursor contamination (Yang and Lin, 2001) after which, CBMCs were washed (250xg, 10 mins) twice in physiological saline (pH 7.4). Approximately 95% of CBMCs obtained were lymphocytes as determined from methylene blue stained smears. CBMC aliquots were frozen at -20°C until fatty acid analysis of membrane fractions.

4.2.2 Fatty acid analysis:

Total lipid was extracted with chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxy toluene under oxygen-free nitrogen (Folch *et al.*, 1957). The phospholipids were separated into phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and bands on silica plates using chloroform/methanol/methylamine (respective ratio 65:35:15). The phospholipid bands were detected with a methanolic solution of 2,7-dichlorofluorescein (0.01% w/v) and viewed under ultraviolet light. Lipid standards were included for identification of bands.

The fatty acid methyl ester (FAME) of each phospholipid band was prepared by heating lipid fractions in 4ml of 15% acetyl chloride in methanol at 70°C for 3 hours under oxygen-free nitrogen FAMEs were separated in a gas liquid chromatograph (HRGC MEGA 2 Series, Fisons Instruments, Italy) fitted with a capillary column (25m x 0.32mm ID, 0.5μ film, BP20). Hydrogen was used as a carrier gas, and the injector, oven and detector temperatures were 250°C, 200°C and 280°C respectively. FAMEs

were identified by comparison of retention times with authentic standards and calculation of equivalent chain length values. Peak areas were quantified by a computer chromatography data system (EZChrom Chromatography Data System, Scientific Software, Inc., San Ramon, CA).

4.2.3 Statistical analysis of the data

Results are expressed as mean, standard error, median and interquartile range. The significance of the difference between the relative percentage membrane fatty acid composition of term, preterm and DNI samples were analysed using the Kruskal-Wallis method for non-parametric data, and expressed using the Dwass-Steel-Chritchlow-Fligner, all pairwise comparison (StatsDirect, statistical software 2006, version 2.5.5). The relationship between AA and birthweight and between DHA and gestational age was given by Spearman's rank correlation for non-parametric measures (r). Significance was calculated using the t-test based on standard deviation between r and zero StatsDirect, statistical software 2006, version 2.5.5.

4.3. Results

Table 4.1: Comparison of the long chain polyunsaturated fatty acid composition of the phosphatidylethanolamine (PE) fraction from term, preterm and DNI CBMCs.

PE fraction	Mean fatty acid percent ± SE (Median and interquartile range)			Kruskal-Wallis test for significance (p value)		
	Term (n=9)	Preterm (n=10)	DNI (n=9)	Term & Preterm	Term & DNI	Preterm & DNI
16:0	**5.4 ± 0.18** 5.3:5.1-5.6	**6.2±0.6** 6.3: 4.5-7.7	**4.9 ± 0.3** 5.0:4.1-5.8	NS	NS	NS
18:0	**17.1 ± 0.5** 17:16.5-18.1	**17.1±1.5** 17.5: 14.3-20.2	**14.9 ± 0.33** 15.1:14.5-15.5	NS	<0.05	NS
16:1ω7/11	**0.5± 0.03** 0.5: 0.45-0.53	**0.4±0.06** 0.4: 0.4-0.5	**0.8 ± 0.09** 0.8:0.6-0.9	NS	<0.05	<0.05
18:1ω7/9	**4.5 ± 0.2** 4.4:4.3-4.5	**5.7±0.4** 5.3: 5.1-6.6	**5.1 ± 0.3** 5.05:4.5-5.8	NS	NS	NS
18:2ω6	**1.7 ± 0.1** 1.6: 1.5-1.7	**1.7±0.1** 1.8: 1.5-1.9	**2.9 ± 0.2** 3.0: 2.4-3.3	NS	<0.01	<0.01
18:3ω6	**0.25 ± 0.03** 0.26:0.18-0.27	**0.5±0.14** 0.3: 0.25-0.67	**0.07 ± 0.02** 0.05:0.04-0.12	NS	<0.01	<0.01
20:3ω9	**0.27 ± 0.03** 0.3: 0.2-0.4	**0.11±0.06** 0.03: 0-0.27	**1.05 ± 0.20** 1.1: 0.6-1.6	NS	<0.05	<0.01
20:3ω6	**1.2 ± 0.01** 1.2: 1.2-1.3	**1.02±0.05** 0.95: 0.92-1.21	**1.4 ± 0.08** 1.4: 1.3-1.6	NS	NS	<0.01
20:4ω6	**34.1 ± 0.6** 33.7: 32.5-35.5	**28.8±2.01** 28.7: 23.3-33.6	**31.3 ± 1.2** 31.0: 28.3-34.7	<0.05	NS	NS
20:5ω3	**0.36 ± 0.04** 0.4: 0.2-0.5	**0.15±0.07** 0.00: 0-0.3	**0.15 ± 0.03** 0.1: 0.07-0.2	<0.05	<0.05	NS
22:4ω6	**5.02 ± 0.25** 5.3: 4.4- 5.4	**4.4±0.23** 4.5: 3.8-4.9	**4.06 ± 0.21** 4.3:3.6-4.5	NS	NS	NS
22:5ω6	**1.2 ± 0.10** 1.2: 1.0-1.5	**0.98±0.18** 0.73: 0.63-1.46	**1.03 ± 0.14** 1.0: 0.7-1.2	NS	NS	NS
22:5ω3	**1.3 ± 0.1** 1.1:1.06-1.5	**0.3±0.1** 0.4:0-0.6	**0.9 ± 0.08** 0.9:0.8-1.1	<0.001	NS	<0.01
22:6ω3	**4.9 ± 0.3** 4.8: 4.5-5.5	**2.5±0.24** 2.6: 2.5-2.9	**3.8 ± 0.3** 4.0: 3.1-4.5	<0.001	NS	<0.05

Significant difference was determined by $p<0.05$. 16:0 is palmitic acid, 18:0 is stearic acid, 16:1ω7 is palmitoleic acid, 18:1ω9 is oleic acid, 18:2ω6 is linoleic acid (LA), 18:3ω6 is gamma linolenic acid (GLA), 20:3ω9 is Mead acid, 20:3ω6 is dihomogamma linoleic acid , 20:4ω6 is arachidonic acid (AA), 20:5ω3 is eicosapentaenoic acid (EPA), 22:4ω6 is docosatetraenoic acid (adrenic acid), 22:5ω6 is ω6-docosapentaenoic acid (Osbond acid), 22:5ω3 is ω3-docosapentaenoic acid and 22:6ω3 is docosahexaenoic acid (DHA).

Arachidonic acid (20:4ω6; AA) comprised the largest portion of fatty acid in the PE fraction (table 4.1) in all the groups but was higher in the healthy term ($p<0.05$) and DNI neonates (not significant) compared to the preterm. This means that AA was present in approximately one in three PE molecules situated in the cell membranes of term and DNI CBMCs. The percentage of stearic acid (18:0) was half that of AA and the second highest fatty acid in the PE fraction of term, preterm and DNI neonates (table 4.1).

The relative percentage of DHA (22:6ω3) in PE was up to a tenth less than the amount of AA present. The percentage of EPA (20:5ω3) was almost a hundred times lower than the level of AA, and similar levels were present in the preterm and DNI CBMCs. These are especially noticeable in the levels of AA, DHA, EPA and the essential linoleic acid (LA, 18:2ω6). When DNI values were compared to term, there was no significant difference in the levels of important dietary fatty acids such as AA and DHA, however there was a noticeably lower level of EPA ($p<0.05$) which was comparable to preterm values.

AA was the again the most predominant LCPUFA in the PC fraction (table 4.2), although it level was half that of the PE fraction. Once again preterm neonates showed deficiencies in ω6 fatty acids, with significant differences ($p<0.05$) compared to term neonates in dihomogamma linoleic acid (20:3ω6), AA, adrenic (22:4ω6) and Osbond acid (22:5ω6).

Table 4.2: Comparison of the long chain polyunsaturated fatty acid composition of the phosphatidylcholine (PC) fraction from term, preterm and DNI CBMCs.

PC fraction	Mean fatty acid percent ± SE (Median and interquartile range)			Kruskal-Wallis test for significance (p value)		
	Term (n=9)	Preterm (n=10)	DNI (n=9)	Term & Preterm	Term & DNI	Preterm & DNI
16:0	**35.1 ± 0.3** 35.3: 34.9-35.4	**34.6±2.1** 36.0: 32.9-40.0	**36. 7 ± 0.9** 37.1:35.1-37.5	NS	NS	NS
18:0	**11.9 ± 0.5** 11.4: 11.0-12.1	**13.0±1.4** 12.6: 11.8-16.0	**12.6 ± 1.4** 13.9: 12.7-14.1	NS	NS	NS
16:1ω7/11	**1.7 ± 0.06** 1.7: 1.7-1.8	**1.6±0.14** 1.6: 1.25-1.91	**1.0 ± 0.3** 0.5: 0.4-1.9	NS	NS	NS
18:1ω7/9	**14.1 ± 0.5** 14.2:13.9-14.7	**18.4±1.3** 18.0: 17.4-21.1	**16.4 ± 0.7** 15.8:15.3-16.8	NS	<0.05	NS
18:2ω6	**5.6 ± 0.3** 5.4: 4.9-6.0	**5.7±0.8** 5.9: 3.69-6.67	**4.6 ± 0.5** 4.7: 3.3-5.3	NS	NS	NS
18:3ω6	**0.37 ± 0.03** 0.4: 0.3-0.4	**0.5±0.08** 0.4: 0.32-0.62	**0.1 ± 0.01** 0.1: 0.1-0.1	NS	<0.01	<0.001
18:3ω3	**0.05 ± 0.01** 0.05: 0.04-0.06	**0.04±0.01** 0.05: 0-0.06	**0.06 ± 0.01** 0.07: 0.05-0.08	NS	NS	NS
20:3ω9	**0.2± 0.03** 0.1: 0.1- 0.2	**0.06±0.02** 0.07: 0.06-0.08	**0.8 ± 0.2** 0.8: 0.5-1.05	<0.01	<0.01	<0.01
20:3ω6	**2.2 ± 0.07** 2.2: 2.05-2.4	**1.9±0.2** 2.1: 1.8-2.2	**1.7 ± 0.14** 1.7: 1.5-1.7	NS	<0.05	NS
20:4ω6	**15.1 ± 0.4** 15.2: 14.7-16.1	**12.5±1.05** 12.9: 11.4-14.4	**12.9 ± 0.9** 13.0: 11.5-13.8	<0.05	NS	NS
20:5ω3	**0.28 ± 0.04** 0.3: 0.2 - 0.3	**0.2±0.04** 0.2: 0.1-0.3	**0.12 ± 0.03** 0.08:0.06-0.2	NS	<0.05	NS
22:4ω6	**1.02 ± 0.06** 0.9: 0.9-1.1	**0.8±0.08** 0.9: 0.7-0.94	**0.9 ± 0.08** 0.96: 0.7-1.06	NS	NS	NS
22:5ω6	**0.3 ± 0.03** 0.3: 0.2-0.3	**1.0±0.78** 0.2: 0.13-0.31	**0.27 ± 0.05** 0.3: 0.18-0.36	NS	NS	NS
22:5ω3	**0.3 ± 0.04** 0.3: 0.26-0.3	**0.06±0.04** 0:0-0.12	**0.2 ± 0.04** 0.2: 0.15-0.3	<0.01	NS	<0.05
22:6ω3	**1.7 ± 0.16** 1.6: 1.4-1.7	**1.03±0.11** 1.12: 0.9-1.2	**1.3 ± 0.13** 1.2: 1.09-1.5	<0.01	NS	NS

Significant difference was determined by $p < 0.05$ and NS refers to values which are not significantly different. 16:0 is palmitic acid, 18:0 is stearic acid, 16:1ω7 is palmitoleic acid, 18:1ω9 is oleic acid, 18:2ω6 is linoleic acid (LA), 18:3ω6 is gamma linolenic acid (GLA), 18:3ω3 is α-linolenic acid (ALA), 20:3ω9 is Mead acid, 20:3ω6 is dihomogamma linoleic acid, 20:4ω6 is arachidonic acid, 20:5ω3 is eicosapentaenoic acid, 22:4ω6 is docosatetraenoic acid (adrenic acid), 22:5ω6 is ω6-docosapentaenoic acid (Osbond acid), 22:5ω3 is ω3-docosapentaenoic acid and, 22:6ω3 is docosahexaenoic acid.

Compared to the preterm PE fraction the levels of fatty acids in the PC fraction were more comparable to term values, particularly with regards to the saturated and monounsaturated fatty acids (Table 4.2). The value of LA and ALA in the preterm was not significantly different to term, while the level of GLA was significantly higher ($p<0.01$) to the term PC level.

Interestingly, the preterm Mead acid level was significantly lower than term, and these results will be described in more detail further on. Preterm neonates had significantly lower levels of ω6 fatty acids in the PC fraction compared to term neonates and these included dihomogamma linoleic acid (20:3ω6; $p<0.05$) and AA (20:4ω6; $p<0.01$). The PC fraction of preterm CBMCs also contained relatively lower levels of DHA (22:6ω3; $p<0.01$) and lower levels of EPA (20:5ω3; not significant) than the term PC fraction.

Although the levels of AA and DHA, along with other ω3 and ω6 LCPUFAs in the PC fraction were lower in DNI than term (table 4.2), these differences were not statistically significant. However DNI neonates had significantly lower levels of GLA (18:3ω6, $p<0.01$) than term and preterm neonates and lower levels of dihomogamma linoleic acid (20:3ω6, $p<0.05$) compared to term babies. The EPA (20:5ω3) level of the PC fraction of DNI CBMCs was less than half the level of that of term ($p<0.05$) and was therefore comparable to preterm levels.

Table 4.3: The Mead acid to AA ratio as a marker of EFA deficiency in the PE and PC fractions of preterm and DNI cord blood mononuclear cells compared to term.

Mean fatty acid percent ± standard error						
	PE			**PC**		
	Term	Preterm	DNI	Term	Preterm	DNI
18:2ω6	1.7 ± 0.11	1.7 ± 0.1	2.89 ± 0.23	5.57 ± 0.33	3.72 ± 0.69	4.61 ± 0.47
18:3ω3	N/A	N/A	N/A	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
18:1ω9	4.54 ± 0.19	0.95 ± 0.12	5.06 ± 0.31	14.14 ± 0.51	10.90 ± 1.92	16.37 ± 0.67
Ratio of 20:3ω9/20:4ω6	**0.008**	**0.005**	**0.03**	**0.01**	**0.005**	**0.06**

Where N/A is not applicable due to the trace amounts of the fatty in the lipid fraction that were too small for analysis.

As mentioned previously, LA (18:2ω6) and ALA (18:3ω3) are essential fatty acids (EFAs), so named because they can only be obtained from dietary sources and are the precursors of important LCPUFAs such as AA, EPA and DHA. Mead acid is a marker of EFA deficiency, depicted by its presence in high amounts when levels of LA and alpha linolenic acid (ALA, 18:3ω3) are very low. As mentioned in chapter 1, in the event of a deficit in LA and ALA, oleic acid (18:1ω9) is converted to Mead acid.

Preterm neonates had a similar level of LA to term neonates in the PE fraction. The level of Mead acid was significantly lower ($p<0.01$) than term neonate levels (Table 4.1). Table 4.3 shows that the Mead acid to AA ratio was lower in preterm CBMCs and there was a significantly lower level of oleic acid and Mead acid ($p<0.01$) in preterm compared to term.

On the other hand, the Mead acid level in DNI neonates was significantly higher than that of term neonates ($p<0.05$) thus making the Mead acid to AA ratio higher in the PE fraction of DNI CBMCs compared to term (table 4.3). As a result a correspondingly smaller level of LA was expected in the DNI neonates. However the level of LA was significantly higher in DNI neonates compared to term ($p<0.01$), while the level of oleic acid was higher in DNI CBMCs but not significantly so.

In the PC fraction, preterm LA and ALA were lower than term neonate levels although there was no statistical significance in the difference between the two groups (tables 4.2 and 4.3). Oleic acid (18:1ω9) was also lower in preterm CBMCs, but this difference was not statistically significant. The level of Mead acid (20:3ω9) and therefore the Mead acid to AA ratio was higher in the term than the preterm ($p<0.05$).

The PC fraction of DNI CBMCs contained relatively higher levels of oleic acid (18:1ω9) than term ($p<0.05$) neonates (tables 4.2 and 4.3). However there was no statistical significance in the difference in the levels of LA and ALA between these groups. DNI neonates had higher levels of Mead acid in the PC fraction when compared to term and preterm neonates ($p<0.01$ and $p<0.05$ respectively). Thus there was also a higher Mead acid to AA ratio in the PC fraction of DNI CBMCs.

Overall The PE fraction contained larger amounts of PUFAs and less saturated fatty acids than the PC fraction in all 3 groups of neonates (tables 4.1 and 4.2).

Table 4.4: The correlation of AA and birth weight and DHA and gestational age between term and preterm neonates in the PE and PC membrane lipid fractions.

	Correlation (r values) of AA and DHA with birthweight and gestational age respectively:		**Significance (p values) of correlation:**	
	AA and birthweight (grams)	**DHA and gestational age (days)**	**AA and birthweight**	**DHA and gestational age**
PE	0.5	0.9	<0.05	<0.001
PC	0.4	0.7	NS	<0.001

Where, $r>0$ is positively correlated, $p<0.05$ is significant and NS is not significant.

Leaf *et al.*, 1992, found in term and preterm plasma samples, that there was a significant positive correlation between AA and birthweight, and between DHA and gestational age. Table 4.4 shows that in the PE fraction of term and preterm CBMCs used in this study, there was indeed a statistically significant strong positive correlation between AA and birthweight ($r = 0.5$, $p<0.05$), and between DHA and gestational age ($r = 0.9$, $p<0.001$). In the PC fraction, although not statistically significant, there was an indication of a positive correlation between AA and birthweight ($r = 0.4$), but there is a much stronger correlation between DHA and gestational age ($r = 0.7$, $p<0.001$).

4.4. Discussion

The cell membrane is a dynamic, asymmetric structure sometimes referred to as fluid (Mathews and van Holde, 1990). Seigneuret and Devaux (1984) initially reported that this asymmetry involving the transport of PE and phosphatidylserine (PS) was an energy-dependent enzyme-driven function. Unfortunately, due to the small quantities of cord blood obtained for the present study, PS and phosphatidylinositol (PI) were only available in trace amounts too small for analysis. In unstimulated T-cells, PC and PE

each contain about 38% of total plasma membrane phospholipid AA, while PS and PI contain 20% and 4% respectively, with only 3% available as free AA (Tomita *et al.*, 2004). In this study on neonatal CBMCs, not specifically T-cells, PE and PC contained approximately 34% and 15% AA respectively.

The CBMCs in all three groups showed a predominance of AA in the PE and PC fractions (tables 4.1 and 4.2), with much lower levels of ω3 LCPUFAs. In term neonatal CBMCs for example, DHA represents only 5% in the PE fraction compared to AA with 34%, and only 2% in the PC fraction, with AA at 15%. The preferential selection of AA from circulation may indicate the preferential selection of AA from circulating pools, in order to facilitate growth, development and optimal functioning of the neonatal immune system.

The level of AA in the plasma membranes of cells is important to cell function and in immune cells the membrane plays and integral role in function. Cellular AA is a precursor to eicosanoids such as prostaglandins that are responsible or the regulation of cellular growth, differentiation and homeostasis (Kaur *et al.*, 1999). More recently AA has been shown to give rise to lipoxins that are potent mediators and regulators of inflammatory reactions (Levy *et al.*, 2001 and Ariel *et al.*, 2003).

Alteration in membrane LCPUFAs, were shown to change the cytotoxic function of cytotoxic T-cells, i.e. unsaturated fatty acids increased cytotoxic function, while saturated fatty acids lead to a decrease (Gill and Clark, 1980). AA, more than other LCPUFAs, was shown to increase T-cell mitogenesis which was observed by tritiated thymidine incorporation at low concentrations of 0.1 to 5.0μg/ml AA, as well as by uridine uptake and blast formation (Kelly and Parker, 1979).

Furthermore, unsaturated fatty acids such as AA released from the cell membrane were shown to activate protein kinase C, and thereby actively participate in signal transduction (Sekiguchi *et al.*, 1987). Although this study was carried out on protein kinase C isolated from rat brain, it was shown that in the presence of Ca2+ unsaturated fatty acids, were able to optimally activate the catalytic activity of type III protein kinase C, and to a lesser extent types I and II.

In addition the AA-derived leukotriene-B4 has been reported to induce differentiation and proliferation of haematopoietic stem cells (Chung *et al.*, 2005), which indicates that AA may be intrinsic to the maturation of fetal cells. Leukotriene-B4 also produces a protective anti-apoptotic effect to these cells (Chung *et al.*, 2005).

In preterm neonates, the very obvious paucity of AA and other LCPUFAs in the PE and PC fractions may indicate that these neonates are born at a time when they have not fully accreted the available stores of LCPUFAs from the maternal supply. Placental biomagnification shows that fetal plasma AA is much lower at preterm than at term, and that the levels in the mother are higher (Crawford *et al.*, 2003). Although at this stage of development it is difficult to call this lower level of membrane LCPUFAs a deficiency, as it would be prudent to analyse total lipid content in order to do this.

The fatty acids of the PE and PC fraction of DNI neonates were very comparable to those of term neonates (tables 4.1 and 4.2). This may have been due to the fact that the DNI neonates used in this study were delivered at term, and would therefore have been expected to have as full a complement of immune cells as healthy term babies. DNI neonates born to HIV-positive mothers were previously shown to have low plasma

levels of LA (Agostoni *et al.*, 1998). In this study it was evident that the levels of LA in DNI CBMCs were either significantly higher than (table 4.1) or comparable to (table 4.2) term levels. The levels of AA and DHA, while slightly lower than term levels were not significantly different, and were therefore also comparable to term levels. This data may indicate that anti-retroviral prophylaxis did not affect the overall distribution of fatty acids within the major phosphoglycerides of the plasma membranes of DNI CBMCs. However this is not a conclusive finding as the fatty acid composition of PS and PI would have to be assessed as well as the comparative diets of the HIV-negative and HIV-positive mothers. Furthermore, the diet of HIV-positive pregnant women residing in the UK with first world healthcare may be different to HIV-positive pregnant women in rural Africa, where anti-retroviral prophylaxis is at a premium.

The major differences in fatty acid composition between term and DNI neonates, was that DNI neonates had significantly lower levels of EPA compared to term levels (tables 4.1 and 4.2). In fact DNI neonates had less that 50% of the levels of term CBMCs. Although EPA was not a major contributor to even healthy term CBMC fatty acid composition, this paucity may be of some importance to the immune cell function of term CBMCs. EPA is a mediator of regulators of inflammatory reactions (Serhan, 2005 a and b).

The fact that more AA was present in the PE fraction than the PC (tables 4.1 and 4.2), may account for the need of AA in signal transduction (Sekiguchi *et al.*, 1987) as well as to the curvature of the membrane. This means that the cytoplasmic face of the membrane would have more unsaturated fatty acids which would facilitate the curve or bend of the membrane. Matos *et al.*, 1990, showed that the phospholipids located on the

cytoplasmic face of rat neonatal cardiomyocytes were rich in AA and stearic acid (18:0).

As mentioned previously Mead acid is a biochemical marker of EFA deficiency, i.e. a deficiency in LA and ALA. In this study, preterm neonates had similar levels of LA and ALA to term neonates, and consequently significantly lower levels of Mead acid in the PC fraction ($p<0.05$) and PE fraction ($p<0.01$). Therefore the Mead acid to AA ratio in these fractions was less than term levels (table 4.3). These findings indicate preterm neonates did not have an EFA deficiency at the time of birth. However, the longer the fetus remains *in utero* until full term, the more nutrients can be accreted. Thus while this may not point to a deficiency, it certainly does mean that at birth the immune cells and all their associated functions may well be compromised in the preterm neonate.

DNI neonates on the other hand showed a significantly higher level of Mead acid and therefore a higher Mead acid to AA ratio than term neonates. This would seem to indicate a deficiency in EFAs but for the highly comparable level of LA and ALA between term and DNI CBMCs. In fact, DNI neonates had a significantly larger percentage of LA than term neonates in the PE fraction ($p<0.05$, table 4.1) and the PC fraction ($p<0.01$, table 4.2). Interestingly, while DNI neonate CBMCs had quite comparable percentages of oleic acid compared to term neonates, the levels of dihomogamma linoleic acid (20:3ω6) were lower in the PE fraction than term (not significant) and significantly lower in the PC fraction ($p<0.05$, table 4.2). As dihomogamma linoleic acid is a product in the pathway of synthesis from LA to AA (figure 1.3), this further illustrated that the high levels of Mead acid were not conclusive EFA deficiencies, as LA was not being converted to dihomogamma linoleic acid. This may also imply that the AA present in the neonate was primarily procured from

circulation rather than *de novo* synthesis. It is plausible that mitochondrial and microsomal damage due to exposure to anti-retroviral prophylaxis such as Zidovudine (Poirier *et al.*, 2003) may have damaged desaturase mechanisms responsible for converting LA and ALA to AA and DHA, and activated instead the oleic acid to Mead acid chain. This is purely speculative, and can only seriously be considered if liver microsomal tests were carried out on the neonates.

Finally, the data established in this experiment showed that there was a strong positive correlation between AA and birthweight (table 4.4). This data is in agreement with data presented by Leaf *et al.* (1992) on the correlation of these same variables in neonatal plasma samples. At about 29 to 34 weeks of gestation, the fetus begins to put on subcutaneous (white) fat, so that a 36 week old fetus growing normally, would seem plumper than a 30 week old fetus (Moore and Persaud, 1998). Therefore if a normal-weight fetus is born preterm, it is preterm by date, as opposed to preterm by weight (Moore and Persaud, 1998). However due to placental insufficiency for example, term and preterm neonates may be born small for date, and would therefore have accumulated less subcutaneous fat than a normally growing fetus. The level of plasma membrane AA is positively correlated with this weight, and adds another dimension to the biomagnification of fetal AA, in that weight and not only gestational age is a factor in the level of AA on the fetal side of the membrane, making maternal diet and placental provision key elements in the accretion of AA.

Similar to the findings of Leaf *et al.*, (1992) there was also a strong positive correlation between DHA in neonatal CBMCs and gestational age at birth. Given that the levels of DHA in the CBMCs were lower than AA (tables 4.1 and 4.2) this may indicate that AA rather than DHA was preferentially selected due to the nature of the cells and their

function. However, in the brain, neural and visual cells, DHA may be accreted in higher quantities at earlier time periods during gestation due to the specific requirements of these cells.

4.5 Conclusion

This study provided novel descriptive and comparative data on the fatty acid profiles of the PE and PC membrane fractions of term, preterm and DNI CBMCs. Overall it has shown deficiencies in LCPUFA content in the PE and PC fractions of preterm and DNI CBMCs compared to term, as well as significant positive correlations between AA and birthweight and DHA and gestational age. It was also found that Mead acid was an unreliable marker for EFA deficiency in DNI cord blood mononuclear cells.

The differences seen in the preterm and DNI fatty acid compositions when compared to term may contribute to the poor immunocompetence reported by others. However, in order to make a complete assessment of the fatty acid status of preterm and DNI CBMCs compared to term, it is of importance to analyse the remaining phospholipid and sphingolipid fractions of the immune cell membranes. This data in relation to the data on immune subset levels (chapter 3) may be useful in order to make informed decisions about antenatal LCPUFA supplementation trials.

Chapter 5

Correlation between CD antigen-defined cord blood mononuclear cells and membrane fatty acid composition

5.1 Introduction

It has been well documented that preterm and DNI neonates are at greater risks of immune complications than term neonates (Wilson *et al.*, 1985, Juretic *et al.*, 2001, Clerici *et al.*, 2000 and Kind *et al.*, 1997). It is also known that preterm and DNI neonates may not have received sufficient amounts of LCPUFAs via the placenta due to short gestation or low maternal levels (Crawford *et al.*, 2003 and Agostoni *et al.*, 1998). This study has thus far determined that preterm and DNI neonates have lower numbers of T-lymphocyte subsets than term neonates (Chapter 3) and further that preterm and DNI neonates have deficiencies in important mononuclear cell fatty acids including AA and DHA (Chapter 4). However it is not known how these deficiencies in LCPUFAs impacts on the maturation of the fetal immune system.

The literature indicates that poor pregnancy outcomes and compromised immune system maturation are associated with nutrition and may be reversed by nutritional intervention, although such trials were mainly on the beneficial effects of multivitamin supplementation. In a study of 28 000 Sudanese pre-school children it was noticed that there was an association between undernutrition and diarrhoea and respiratory infections (Kossmann *et al.*, 2000). Vitamin supplementation trials in Tanzania showed that multi-vitamin supplementation in 1075 HIV-infected pregnant women decreased the risk of low birthweight and preterm birth while increasing maternal CD3, CD4 and CD8 counts (Fawzi *et al.*, 1998). Studies by Semba *et al.* (1993) showed that in children with immune abnormalities in T-cell subsets, supplementation with vitamin A reversed these

abnormalities, resulting in higher CD4:CD8 ratios and higher levels of naïve CD4 T-cells.

The effect of antenatal supplementation with LCPUFAs on pregnancy outcome and neonatal immune system maturation is yet to be established. Thus the aim of the following work was to determine an association between individual fatty acids and immune cell subsets of term, preterm and DNI CBMCs studied in this thesis.

5.2 Methods

The data presented in this chapter is a statistical correlation of the data obtained and discussed in chapters 3 and 4. The fatty acid data from the PC and PE fraction of 3 term, 4 preterm and 8 DNI neonate cord blood lymphocyte cell membranes, were correlated with immunophenotype subset cell levels of the same babies. The sample number of neonates is small as this assessment was done retrospectively, and it was not always possible to match neonates for GC and flow cytometric data.

5.2.1 Data analysis

The correlation between fatty acid percentages and immune subset levels (chapter 5) was given by Spearman's rank correlation for non-parametric measures (r). Significance was calculated using the t-test based on standard deviation between r and zero using StatsDirect, statistical software 2006, version 2.5.5. The significance value ($p<0.05$) rejects the null hypothesis that there is a mutual independence between fatty acid level and immune subset level.

5.3 Results

The results are given by the statistical correlation (r) and the significance of correlation (p value). Positively correlated values given in tables 5.1 and 5.2 refer to values that are directly proportional to each other (i.e increase or decrease mutually dependent of each other). Negatively correlated variables are inversely proportional (i.e as one increases the other decreases).

Table 5.1 shows the PE fraction of CBMC membranes from term, preterm and DNI neonates where, significant positive correlation ($p<0.01$-0.05) was seen between LA (18:2ω6) and CD4 and CD8 leukocytes as well as T-cells (CD3/4 and CD3/8) and CD8 naïve cells (CD8/RA). Docosapentaenoic acid (DPA – 22:5ω6) was also positively correlated ($p<0.01$-0.05) with CD4 and CD8 leukocytes and lymphocytes as well as CD4 naïve cells (CD4/RA). Docosatetraenoic acid (DTA – 22:4ω6) was significantly positively correlated ($p<0.001$-0.05) with activated or IL-2 responsive leukocytes (CD25/45), T-cells (CD3/25) and CD4 memory cells (CD4/RO). DHA (22:6ω3) was significantly positively correlated ($p<0.05$) with pan T-cells (CD3/45) but especially with CD8 leukocytes (CD8/45) and CD8 T-cells (CD3/8). The only significantly negative association was given by stearic acid (18:0) with CD4/RA ($p<0.05$).

The PC fraction (table 5.2) showed similar positive associations for LA and CD4 and CD8 leukocytes and lymphocytes as seen in the PE fraction. Similar significant positive correlations ($p<0.001$-0.05) were noted between AA and DHA and most of the immune subsets. DHA in particular was strongly significantly correlated with CD45, CD3/45 and CD8/RA ($p<0.001$). In the PC fraction DTA was not strongly correlated with activated cells as seen in the PE fraction but rather positively correlated with all CD4 and CD8 cells ($p<0.01$-0.05). DPA was positively correlated ($p<0.05$) with CD4 T-cells

and leukocytes as well as B-lymphocytes (CD19/45). There were more negative correlations in the PC fraction than in the PE, for example 16:0 (palmitic acid) while positively associated with B-cells ($p<0.05$) was negatively associated with CD8 memory cells. Stearic acid (18:0) was negatively associated with B-cells ($p<0.01$). Mead acid was the most negatively correlated with B-cells, activated leukocytes and T-cells as well as CD4/RO memory cells was 20:3ω9 ($p<0.01$-0.05).

Table 5.1: The correlation of fatty acids in the PE fraction of term, preterm and DNI neonates and level of immune phenotypes in cord blood.

	LCPUFAs in the PE fraction of cord blood mononuclear cells r (correlation coefficient) significance of correlation											
CD Antigens	**16:0**	**18:0**	**18:2n6**	**18:3n6**	**20:3n9**	**20:3n6**	**20:4n6**	**20:5n3**	**22:4n6**	**22:5n6**	**22:5n3**	**22:6n3**
CD45	-0.35 NS	-0.15 NS	0.56 p<0.05	-0.33 NS	-0.12 NS	0.26 NS	0.13 NS	0.04 NS	0.20 NS	0.52 p<0.05	0.35 NS	0.48 p<0.05
CD4/45	-0.04 NS	-0.25 NS	0.69 p<0.01	-0.34 NS	0.19 NS	0.36 NS	0.05 NS	0.16 NS	0.32 NS	0.58 p<0.05	0.31 NS	0.37 NS
CD8/45	-0.33 NS	-0.03 NS	0.55 p<0.05	-0.24 NS	-0.05 NS	0.26 NS	-0.05 NS	-0.05 NS	0.11 NS	0.43 NS	0.34 NS	0.47 p<0.05
CD3/4	-0.04 NS	-0.25 NS	0.69 p<0.01	-0.34 NS	0.19 NS	0.36 NS	0.05 NS	0.16 NS	0.32 NS	0.58 p<0.05	0.31 NS	0.37 NS
CD3/45	-0.18 NS	-0.25 NS	0.64 p<0.01	-0.42 NS	0.15 NS	0.38 NS	0.03 NS	0.08 NS	0.18 NS	0.61 p<0.01	0.44 NS	0.50 p<0.05
CD3/8	-0.39 NS	-0.11 NS	0.55 p<0.05	-0.41 NS	0.14 NS	0.27 NS	-0.17 NS	-0.11 NS	-0.02 NS	0.45 p<0.05	0.39 NS	0.55 p<0.05
CD25/45	0.25 NS	-0.22 NS	0.14 NS	0.17 NS	-0.27 NS	-0.19 NS	0.38 NS	0.51 NS	0.55 p<0.05	0.27 NS	0.002 NS	0.07 NS
CD19/45	-0.26 NS	-0.15 NS	-0.01 NS	-0.03 NS	-0.41 NS	0.09 NS	0.22 NS	0.27 NS	0.11 NS	0.19 NS	0.17 NS	0.16 NS
CD19/25	0.23 NS	0.05 NS	-0.13 NS	0.32 NS	-0.27 NS	-0.1 NS	-0.06 NS	0.39 NS	0.12 NS	-0.03 NS	-0.18 NS	0.03 NS
CD3/25	0.24 NS	-0.28 NS	0.08 NS	0.25 NS	-0.31 NS	-0.27 NS	0.36 NS	0.44 NS	0.66 p<0.01	0.28 NS	-0.07 NS	0.01 NS
CD8/RA	-0.28 NS	-0.08 NS	0.50 p<0.05	-0.20 NS	-0.09 NS	0.20 NS	0 NS	-0.04 NS	0.11 NS	0.38 NS	0.25 NS	0.39 NS
CD4/RA	0.05 NS	-0.47 p<0.05	0.33 NS	-0.23 NS	0.03 NS	0.35 NS	0.01 NS	0.18 NS	0.29 NS	0.52 p<0.05	0.11 NS	0.15 NS
CD4/RO	-0.02 NS	-0.36 NS	0.08 NS	0.23 NS	-0.38 NS	-0.09 NS	0.2 NS	0.34 NS	0.86 p<0.001	0.41 NS	-0.03 NS	0.17 NS
CD8/RO	-0.26 NS	-0.13 NS	0.13 NS	-0.03 NS	-0.15 NS	0.05 NS	-0.12 NS	0.12 NS	0.38 NS	0.44 NS	0.19 NS	0.44 NS

Table 5.2: *The correlation of fatty acids in the PC fraction of term, preterm and DNI neonates and level of immune phenotypes in cord blood.*

	LCPUFAs in the PC fraction of cord blood mononuclear cells r (correlation coefficient) significance of correlation											
CD Antigens	**16:0**	**18:0**	**18:2n6**	**18:3n6**	**20:3n9**	**20:3n6**	**20:4n6**	**20:5n3**	**22:4n6**	**22:5n6**	**22:5n3**	**22:6n3**
CD45	0.27 NS	-0.44 NS	0.54 p<0.05	-0.05 NS	-0.50 p<0.05	0.36 NS	0.71 p<0.01	-0.22 NS	0.58 p<0.05	0.50 p<0.05	0.22 NS	0.74 p=0.001
CD4/45	0.09 NS	-0.09 NS	0.31 NS	0.02 NS	-0.07 NS	0.2 NS	0.57 p<0.05	-0.16 NS	0.65 p<0.01	0.49 p<0.05	0.18 NS	0.62 p<0.01
CD8/45	0.29 NS	-0.33 NS	0.46 p<0.05	-0.03 NS	-0.42 NS	0.28 NS	0.64 p<0.01	-0.15 NS	0.54 P<0.05	0.44 NS	0.33 NS	0.73 p<0.01
CD3/4	0.09 NS	-0.09 NS	0.31 NS	0.02 NS	-0.07 NS	0.2 NS	0.57 p<0.05	-0.16 NS	0.65 p<0.01	0.49 p<0.05	0.18 NS	0.62 p<0.01
CD3/45	0.21 NS	-0.25 NS	0.39 NS	-0.02 NS	-0.17 NS	0.31 NS	0.61 p<0.01	-0.16 NS	0.68 p<0.01	0.57 p<0.05	0.37 NS	0.79 p<0.001
CD3/8	0.22 NS	-0.22 NS	0.29 NS	-0.19 NS	-0.16 NS	0.13 NS	0.44 NS	-0.31 NS	0.50 p<0.05	0.39 NS	0.31 NS	0.63 p<0.05
CD25/45	-0.02 NS	-0.37 NS	0.58 p<0.05	0.51 p<0.05	-0.68 p<0.01	0.42 NS	0.47 p<0.05	0.34 NS	0.22 NS	0.06 NS	-0.15 NS	0.39 NS
CD19/45	0.53 p<0.05	-0.63 p<0.01	0.57 p<0.05	0.08 NS	-0.69 p<0.01	0.53 p<0.05	0.47 p<0.05	-0.03 NS	0.31 NS	0.50 p<0.05	-0.08 NS	0.36 NS
CD19/25	-0.09 NS	-0.11 NS	0.35 NS	0.35 NS	-0.41 NS	0.19 NS	-0.13 NS	0.23 NS	-0.21 NS	-0.39 NS	-0.61 p<0.05	-0.13 NS
CD3/25	-0.13 NS	-0.35 NS	0.58 p<0.05	0.44 NS	-0.73 p<0.01	0.42 NS	0.44 NS	0.38 NS	0.22 NS	0.09 NS	0 NS	0.38 NS
CD8/RA	0.31 NS	-0.36 NS	0.51 p<0.05	0.002 NS	-0.41 NS	0.34 NS	0.69 p<0.01	-0.08 NS	0.5 p<0.05	0.42 NS	0.37 NS	0.77 p<0.001
CD4/RA	0.07 NS	-0.21 NS	0.40 NS	0.17 NS	-0.14 NS	0.48 p<0.01	0.34 NS	0.29 NS	0.56 p<0.05	0.39 NS	0.53 p<0.05	0.61 p<0.01
CD4/RO	-0.42 NS	-0.11 NS	0.59 p<0.05	0.11 NS	-0.64 p<0.05	0.33 NS	0.26 NS	0.12 NS	0.25 NS	0.09 NS	-0.06 NS	0.21 NS
CD8/RO	-0.50 p<0.05	-0.07 NS	0.37 NS	0.06 NS	-0.35 NS	0.16 NS	-0.07 NS	-0.02 NS	0.22 NS	-0.13 NS	-0.05 NS	0.13 NS

5.4 Discussion

It is well-established that preterm and DNI neonates have lower levels of immune cell subsets than healthy term neonates at the time of birth. This is reflected in their post-natal susceptibility to non-specific infections. In preterm neonates, prolonged inflammation in response to infection as well as injury is less well explained.

This chapter has shown that the results obtained for immune subset levels and fatty acid composition of the PE and PC fractions of the membranes of these cells are related in mutually dependent ways. At this stage it is difficult to give a distinctive cause and effect relationship between the fatty acids and immune cell subset levels. However the information is important in that it reveals that certain fatty acids may be selectively incorporated into the PE and PC fractions of specific subsets of immune cells. For example tables 5.1 and 5.2 indicates that the level of LA in the PE and PC fraction of all CD4 and CD8 lymphocyte membranes is directly proportional to the levels of those cells in cord blood. It is known that dietary supplementation of omega 3 and omega 6 fatty acids to guinea pigs showed that these LCPUFAs were mainly incorporated into the inner leaflet of red blood cell membranes (Poschl *et al.*, 1999). Furthermore, lymphocytes preferentially incorporate omega 6 fatty acids such as AA and linoleic acid (LA) during growth and proliferation *in vitro* (Calder, 1994 and Rode *et al.*, 1992).

Although it cannot be assumed that the fatty acids correlated with immune subset levels play an important role in the cell functioning, the positive correlation of an LCPUFA such as AA with CD4 and CD8 lymphocytes and CD8 naïve cells (table 5.2) may relate to more specific functions such as signalling or acting as the precursor to eicosanoids. Eicosanoids are known to be important mediators of immune function and regulators of platelet aggregation, blood clotting, leukocyte chemotaxis and inflammatory cytokine

production (Calder, 2003). Prostaglandins are eicosanoids that regulate cellular growth, differentiation and homeostasis (Kaur *et al.*, 1999). Upon inflammatory stimulation, AA is released from the cell membrane by phospholipase A_2 and converted to PGH_2 by the cyclooxygenase enzymes (COX-1 and COX-2) (Harris *et al.*, 2002) together with specific isomerases (Tilley et al., 2001). PGH_2 is then converted by cell specific prostaglandin synthases into various prostaglandins (PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$) (Kaur *et al.*, 1999) and receptors all of which work in sites close to their synthesis.

PGE_2 is one of the most studied prostaglandins, and through experiments using knock-out mice for COX-1 and COX-2, 5-lipoxygenase and prostaglandin receptors, PGE_2 has been shown to be a potent modulator of immune function (Calder, 2003 and Harris *et al.*, 2002). Goetzl *et al.*, (1995) showed that PGE_2 may protect immature CD4+CD8+ double positive thymocytes from apoptosis and Rocca *et al.*, (1999) suggested that COX isoenzymes might play a part in regulating lymphocyte development in the thymus.

In addition AA gives rise to lipoxins that are potent mediators of inflammatory actions by serving as inhibitors of proinflammatory gene activation and infiltration of polymorphonuclear (PMN) cells into injury sites (Levy *et al.*, 2001 and Ariel *et al.*, 2003). Lipoxins are also responsible for inducing the uptake of apoptotic PMN cells by macrophages at the site of injury, therefore initiating the 'clean up' mechanism after inflammation (Godson *et al.*, 2000 and Mitchell *et al.*, 2002). The AA-derived leukotriene-B4 has also been shown to induce differentiation and proliferation of CD34+ haematopoietic stem cells *ex vivo*, as well as produce a protective anti-apoptotic effect to these cells (Chung *et al.*, 2005).

Interestingly, CD25/45 and CD3/25 were correlated with only DTA in the PE fraction and with LA, GLA (18:3ω6) and AA in the PC fraction. It is not known whether a combination of these fatty acids in the PE and PC fractions facilitates the surface expression or responsiveness of IL-2 (CD25) receptors.

Rouquette-Jazdanian *et al.*, (2002) have shown that although lipid rafts in the cell membrane (which contain the proteins involved in T-cell signalling) are predominantly comprised of saturated fatty acids and cholesterol, the surrounding membrane is made up largely of highly unsaturated fatty acids (Rouquette-Jazdanian *et al.*, 2002). However there was no significantly positive correlation between saturated fatty acids (16:0 and 18:0) and T-cells.

Mead acid in the PC fraction was negatively correlated with all B-cells, leukocytes, activated (CD25+) leukocytes and T-cells and CD4 memory cells (CD4/RO). The activated cells are IL-2 responsive and therefore ready to proliferate, whereas CD4/RO memory cells arise from exposure to antigen. It is interesting to note that while Mead acid is significantly negatively correlated with these potentially expanding cell subsets, LA is significantly positively correlated with them (table 5.2). As Mead acid is considered the marker for LA deficiency (Holman, 1977), it seems that these particular cell subsets have a preferential incorporation of LA which is reflected in the low Mead acid levels.

In recent years DHA has emerged as a pro-resolving mediator in inflammation, and although previously DHA was known to be an anti-inflammatory agent (Calder *et al.*, 2002 and Mori and Beilin, 2004) the mechanisms of actions were not clear. It has been shown that omega 3 LCPUFAs such as DHA and EPA give rise to D-series and E-series

resolvins respectively which prevent inflammatory cytokine production as well as controlling the migration of PMN cells into the site of injury (Serhan, 2005a,b and Serhan, 2002). Marcheselli *et al.* (2003) showed that another novel DHA-derived mediator 10,17S-docosatriene was produced after brain injury and also prevented PMN cell infiltration and proinflammatory gene activation, but as importantly provided a neuroprotective response. DHA seems to be specifically incorporated into the membranes of T-cells, leukocytes and CD4 and CD8 naïve cells in the PC fraction (table 5.2) as well as with leukocytes and CD8 cytotoxic cells in the PE fraction (table 5.1). Interestingly EPA (20:5ω3) was the only LCPUFA not significantly associated with any of the cell subsets, and in fact in the PC fraction was weakly negatively correlated with T-cells.

5.5 Conclusion

This preliminary work has determined that there are significant associations between particular fatty acids and immune cell subsets. It seems likely that these fatty acids are selectively incorporated to specific membrane species of phospholipids in specific immune cell subsets. However at this stage it can only be hypothesized that these fatty acids play key roles in the functioning of immune cells, as detailed information is required regarding immune subset levels and fatty acids in other phospholipids and sphingolipid fractions in CBMC membranes.

The results outlined in this chapter provide a gamut of potential experiments into the effects of LCPUFAs on immune system maturation and function. Such information may be vital in the preparation for antenatal and postnatal LCPUFA supplementation to improve pregnancy outcomes.

Chapter 6

Effects of fatty acids on cord blood mononuclear cell survival *in vitro*

6.1. Introduction

Thus far compared to term neonate CBMCs, preterm and DNI CBMCs, showed lower levels of certain LCPUFAs including AA and DHA, lower numbers of immune cell subsets as well as significant correlations between fatty acids and immune subset levels (chapters 3 to 5). The CBMC membrane of term, preterm and DNI neonates were dominated by AA, suggesting an important role for the LCPUFA in the structure and function of the immune cells.

LCPUFAs have been shown to inhibit the growth of cancer cell lines (Grammatikos *et al.*, 1994), play a part in cell signalling (Rouquette-Jazdanian *et al.*, 2002) and regulate inflammatory reactions at the cellular level by reducing PMN infiltration into the site of injury and inhibiting proinflammatory gene activation (Marcheselli *et al.*, 2003). Studies on the effects of LCPUFAs on lymphocytes in culture have provided important, albeit conflicting findings. Kelly and Parker (1979) have shown that AA more than any other LCPUFA had increased tritiated thymidine uptake by human peripheral blood lymphocytes, while Zurier *et al.*, 1999 showed that LCPUFAs have an inhibitory effect on thymic lymphocyte proliferation, with AA having the smallest effect and DHGLA the largest effect. In contrast other studies have determined that LCPUFAs, in particular AA and DHA, are the most potent inhibitors of mitogen stimulated human T-cell proliferation and thymic lymphocyte proliferation (Søyland *et al.*, 1993 and Rotondo *et al.*, 1996).

The aim of the following work was to elucidate the effect of the LCPUFAs AA and DHA on mononuclear cells in culture. The response of the cells to AA and DHA was gauged by cell survival and activity as well as proliferation in the presence of a mitogen.

6.2 Methods

6.2.1 Time-dose experiments with AA and DHA-supplemented media

CBMCs were isolated from term, preterm and DNI neonates as described in chapter 2, sections 2.3 and washed in RPMI 1640 (Sigma, appendix 2) for 10min at 250xg. The cells were then resuspended to a concentration of 5 x 10^4 cell/100µl in fresh unsupplemented RPMI 1640. Small numbers of cells were used due to the limited volume of cord blood available. Further as a result of the requirement of between 20 to 30ml of whole blood required for experimentation, the number of babies used was limited. Thus most experiments consisted of multi-replicate tests on one or two neonates initially. Preterm neonates cord blood was at a premium, and therefore limited the number of experiments, while more blood was obtained for DNI and term experiments.

Cell suspensions of 100µl were added to several wells of a 96-well culture plate. The plates were then centrifuged at 1000 RPM for 10 minutes. The supernatants were removed. The cells in each well were resuspended in RPMI 1640 (Sigma) supplemented with fetal calf serum (FCS, Sigma), penicillin (5000units/ml, Sigma) and streptomycin (0.9% in sodium chloride, Sigma) and concentrations of either AA (32 to 256µM) or DHA (30 to 244µM). The viability of the CBMCs incubated at the different concentrations of AA or DHA for 1 to 24 hours was assessed using the MTS assay (described below).

6.2.1.1 MTS assay

Since facilities were not available for the use of radioactive materials, the aqueous non-radioactive one-solution assay (MTS) was used to colorimetrically assess the proliferation of CBMCs that were incubated with and without AA. As described in the chapter 2, MTS (CellTiter 96^{R} Aqueous ONE Solution Cell Proliferation Assay, Promega) is a colorimetric method, which can be used to determine the number of viable cells in proliferation assays. At the end of timed experiments MTS solution (37^{o}C) was added to each well at a concentration of 20µl/100µl of medium in each well. The plates were further incubated for 1 hour at 37^{o}C, and the viability of the cells was obtained spectrophotometrically at a wavelength of 492nm.

6.2.2 Lymphocyte subset counts using flow cytometric analysis

In order to analyse the lymphocyte proliferation in culture medium containing AA or DHA, cell suspensions were prepared in 96-well plates as described above. A T-cell mitogen phytohaemagglutinin (PHA, 5µg/ml) was added to the medium and the cells were cultured for 3 days prior to being counted by flow cytometric analysis using the four-colour flow cytometry the EPICS XL-4 flow cytometer (Beckman Coulter). The fluorochrome-conjugated monoclonal antibodies (IOtest; Beckman Coulter) raised against lymphocyte cluster of differentiation (CD) antigens used were: T-cell, FITC-CD4 (clone 13B8.2); ECD-CD3 (clone UCHT1); PC5-CD8 (cloneB9.11); memory/naïve, PE-CD45/RA (clone ALB11); ECD-CD45RO (clone UCHL1); B-cell, FITC-CD19 (clone J4.119); pan-leukocyte, PE-CD45 (clone J33); and activated (IL-2-expressing), PC5-CD25. Appropriate isotype controls for IgG1 Abs (all FITC-,PE-and-ECD-conjugates) and IgG2a (PC5-conjugates were clones 79.1Mc7 and U7.27 respectively). Prior to analysis, 100µl of cell suspensions in individual wells was

incubated with combinations of monoclonal antibodies (described above) for 10 minutes. The absolute counts (cells/μl) for each phenotype obtained.

6.3 Results

6.3.1 The time and dose effect of AA and DHA on CBMCs

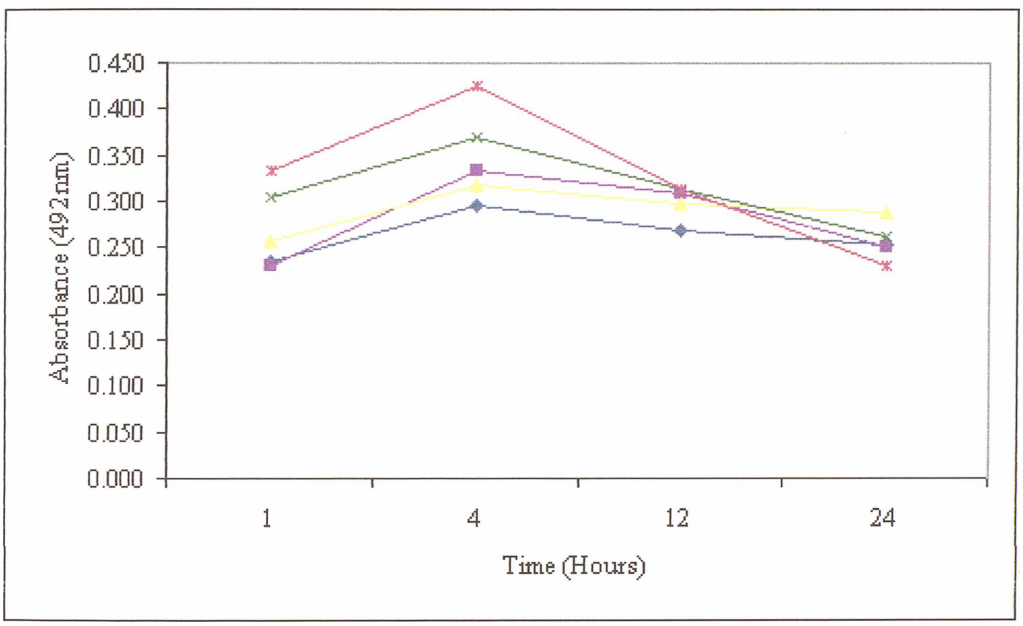


Figure 6.1: The incubation of term CBMCs with AA. The concentrations of AA added to culture media were ■ 0, ■32, ■64, ■128 and ■256μM for 1 to 24 hours at 37°C.

The effects of different doses of AA and DHA on term preterm and DNI CBMCs were tested by the presence of viable CBMCs remaining in culture over periods of time. This was accomplished using the colorimetric MTS assay as described previously.

Figure 6.1 shows the viability of term neonate CBMCs to concentrations of AA added to the media (0 to 256μM), where 0μM meant that the culture medium did not contain exogenous sources of AA. The data indicated that there was a very slight difference in viability between the concentrations; however the cells in all the treatment media followed a similar trend. At all concentrations a peak in optical density (OD) occurred

at 4 hours, before slightly tapering off. The activity seemed to be higher in cells cultured in media containing 256μM AA or 80μg of AA per ml. Spectrophotometrically, the cells cultured in control media (without AA) showed the lowest level of activity or OD.

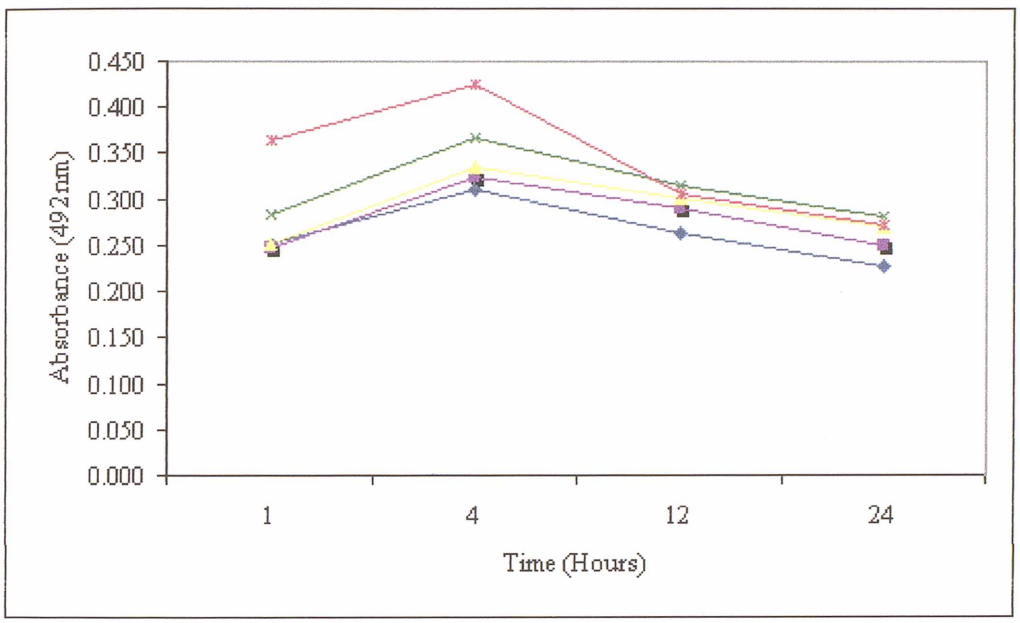


Figure 6.2: Term CBMCs were incubated with DHA for 1 to 24 hours at 37°C. DHA at concentrations of ■ 0, ■30, ■61, ■122 and ■244μM were added to culture media.

Figure 6.2 showed that CBMCs of term neonates cultured with DHA (0 to 244μM) also showed very little difference between the concentrations as well as peaking at 4 hours of culture. As with cells cultured with AA, the OD peaked at 244μM DHA or 80μg of DHA per ml. Term neonate CBMCs cultured in media without DHA (0μM) showed the lowest activity level.

Preterm CBMCs were cultured under the same conditions as the term CBMCs, however due to the small volumes of whole cord blood collected and therefore the limited number of cells, the preterm CBMCs were cultured in media containing AA and DHA for 4, 12 and 24 hours only.

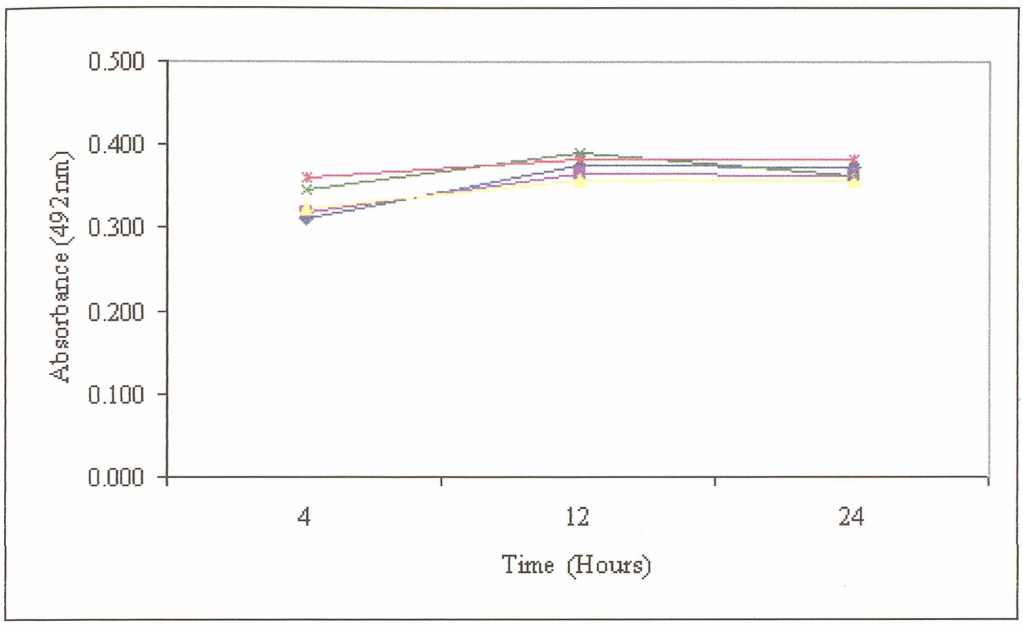


Figure 6.3: Incubation of preterm CBMCs with AA showed very little difference in optical density at all concentration. AA was added to culture media at ■ 0μM; ■ 32μM; ■ 64μM; ■ 128μM; ■ 256μM for 4 to 24 hours at 37°C.

Figure 6.3 shows that there was an even smaller difference in cellular activity between the concentrations of AA and control media as measured by the MTS assay, when compared to term neonate CBMCs. The preterm cells showed a very slight peak in activity at 12 hours of culture.

A similar trend in the activity of preterm CBMCs can be reported when the cells were cultured in media containing 0 to 244μM DHA (figure 6.4). Although there was no difference in the OD of the cells cultured in media without DHA or media with low levels of DHA, there was a definite peak of OD at 12 hours in culture. Maximal activity was observed in preterm CBMCs cultured with 244μM DHA.

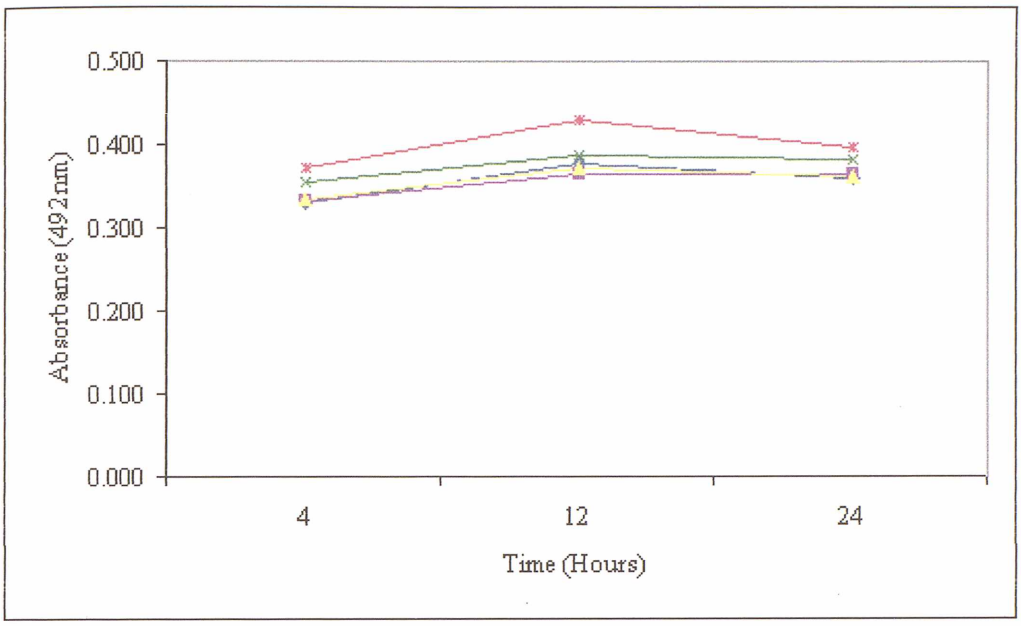


Figure 6.4: Preterm CBMCs were cultured in media containing DHA for 4 to 24 hours. Cell culture media was supplemented with DHA at concentrations of ■ 0μM; ■ 30μM; ■ 61μM; ■ 122μM; ■ 244μM.

For reasons of safety CBMCs isolated from DNI neonate cord blood could not be cultured in the category 1 laboratory (for the culture of non-infective cell lines) until the retroviral status of the neonates was known. As these tests would have taken a minimum of 6 months to be confirmed, the CBMCs were cultured in a standard hospital haematology laboratory for 4 hours. The culture time of 4 hours was chosen due to the optimal OD results obtained for term neonates CBMCs cultured in 256μM AA and 244μM DHA (figures 6.1 and 6.2). CBMCs of 5 DNI neonates were tested for viability in media containing either 256μM AA or 244μM DHA. Control media did not contain AA or DHA.

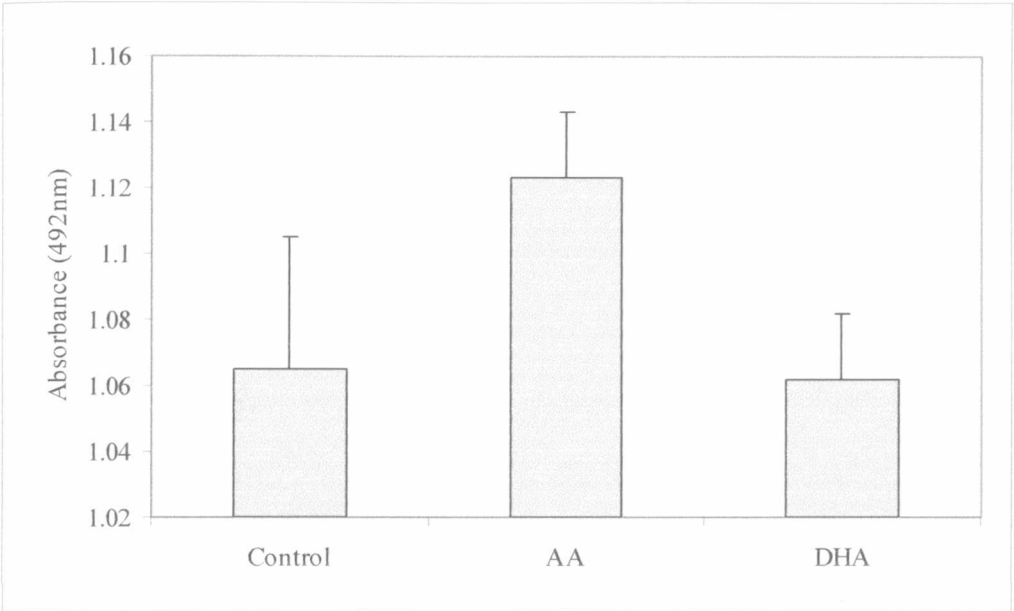


Figure 6.5: The activity given by absorbance values of term DNI CBMCs to media supplemented with 256μM AA and 244μM DHA for 4 hours at 37°C.

The DNI CBMCs showed a similar trend to term CBMCs, in that there were higher absorbance values in cells cultured in medium containing 256μM AA. The DNI CBMCs cultured in medium containing DHA showed similar absorbance values to those in unsupplemented culture media. However there was no significant difference between the means for AA and DHA-unsupplemented cells and cells cultured with AA and DHA. There was also no significant difference between the mean absorbance of cells cultured with AA and those cultured in DHA for 4 hours at 37°C.

6.3.2 The effect of AA on lymphocyte subset counts *in vitro*

Having established viability of CBMCs cultured in media containing AA over short periods of time, it was of importance to determine the effect of such culture media on the proliferation of immune cell subsets over a longer culture period i.e. 3 days.

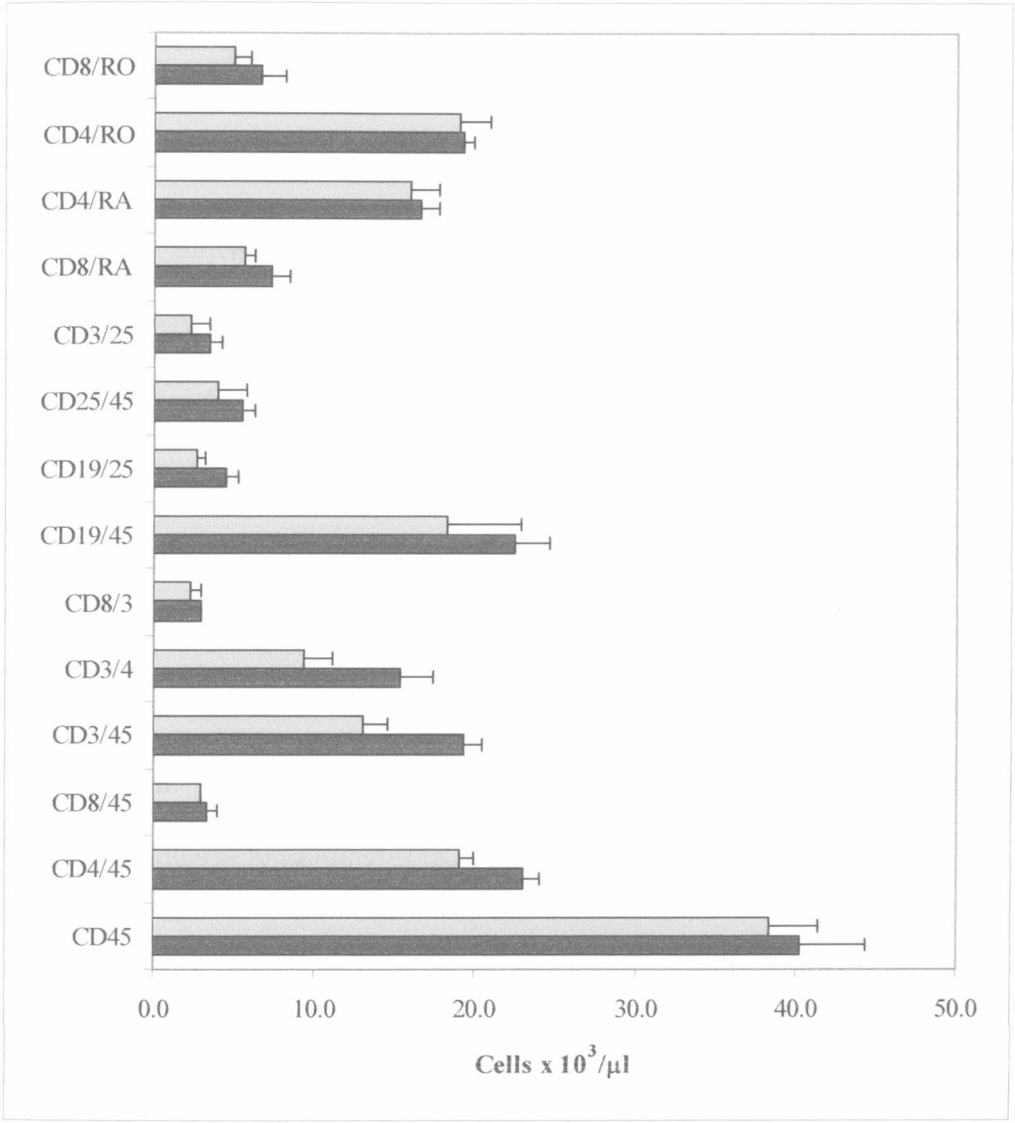


Figure 6.6: Term CBMCs were cultured in medium containing 256µM AA and phytohaemagglutinin for 3 days to ascertain immune subset levels. CD4 and CD8 cells in the CD3 and CD45 subsets, B cells (CD19/45) and activated B-cell (CD19/25) populations and activated leukocyte (CD25/45) and T-cells (CD3/25) were quantified; where (■) represents AA-supplemented cells and (■) represents unsupplemented control cells.

There was an overall increase, albeit small, in the numbers of T-cells and B-cells that were cultured in media containing AA compared to unsupplemented media. Relative to other subset counts larger differences were seen in the T-cell population as a whole (CD3/45), but more especially in the CD4 (Helper) T-cell population (CD3/4).

6.4 Discussion

Previous work on the effects of LCPUFAs on lymphocytes *in vitro* has shown LCPUFAs to be inhibitors rather than stimulators of lymphocyte proliferation. Zurier *et al.*, (1999), showed that unsaturated fatty acids lowered T-cell proliferation in a dose-dependent manner, with AA being the least inhibitory. Rotondo *et al.*, (1996) showed a concentration dependent inhibition of thymic lymphocyte proliferation, in the range of 1-100μM, for DHGLA, EPA and AA. Calder and Newsholme (1992) found that all polyunsaturated fatty acids they tested, including AA and DHA, caused a decrease in the concentration of IL-2 secreted by T lymphocytes. The secretion of IL-2 by T-lymphocytes as well as the expression of IL-2 receptors on lymphocytes is essential to proliferation. IL-2 receptor expression has been correlated with production of IL-2 in human peripheral lymphocytes (Karsten *et al.*, 1994).

At birth the human immune system is immature and contains very high levels of naïve (CD45RA) cells (Neubert *et al.*, 2000). The naïveté of CD4 cells together with lower antigen and mitogen-specific T-cell proliferation, and lower cytokine production upon stimulation, is beneficial in stem cell transplantation (Cohen and Madrigal, 1998). PHA is widely used as a mitogen in the culture of lymphocytes and studies have shown that PHA induces the stimulation of CD4+ T-cells and thereby shifts the CD45RA cells into CD45RO cells in CBMCs (Neubert *et al.*, 2000 and Maccario *et al.*, 1993). However Zhao *et al.* (2002) showed that fetal and neonatal T-cells responded poorly in comparison with adult cells to stimulation with PHA. This lower lymphocyte proliferative response was more pronounced in very low birth-weight neonates (Bussel *et al.*, 1988). Neonate cord blood B and T cells are immature and T-cells are naïve with suppressor activity for B-cell function and with little helper function (Tucci *et al.*, 1991) or in some cases no helper function (Clement *et al.*, 1990). Furthermore, CD34+ cord

blood cells are morphologically immature compared to peripheral blood and bone marrow cells (Mikami *et al.*, 2002) and the expression of immature T and B cell markers have been shown to be higher in preterm and term neonates exposed to antenatal hypoxic stress (Wilson *et al.*, 1985).

In light of the above, it was of interest to determine how neonatal cord blood immune cells responded to supplementation with the LCPUFAs AA and DHA *in vitro*. Thus far it had been established that the preterm and DNI neonates compared to healthy neonates born at term, have suboptimal levels of LCPUFAs such as AA and DHA (chapter 4), as well as deficiencies in immune subset levels at the time of birth (chapter 3).

Figures 6.1 and 6.2 showed that the supplementation of term CBMCs in culture media with AA and DHA respectively, did not produce large differences in optical density with concentration and time. However, it was noticed that a peak in optical density was obvious at 4 hours of culture at all concentrations including 0μM AA and DHA. Preterm CBMCs in culture showed a peak in optical density at 12 hours of culture at all concentrations including 0μM AA and DHA (figures 6.3 and 6.4). Furthermore DNI CBMCs showed higher OD values when cultured for 4 hours in media containing 256μM AA compared to DHA-supplemented media and unsupplemented (control) media (Figure 6.5). The peak in OD values seemed to be more time than concentration dependent (figures 6.1 to 6.4).

As described previously the OD value is a spectrophotometric measure of the number of viable (living) cells within the culture. The product used to determine viability was MTS (Promega) which is added in liquid form to the cell culture medium after the appropriate culture time. The MTS tetrazolium compound is bioreduced into a coloured

formazan product by NADPH or NADH, which is produced by mitochondrial dehydrogenase enzymes present in metabolically active cells. Formazan is soluble in tissue culture medium such as RPMI. The absorbance of the formazan was measured spectrophotometrically at 492nm and is directly proportional to the number of metabolically active cells in the culture (Cory *et al.*, 1991).

The cell cycle takes 24 hours of which 6 to 8 hours is concerned with the S-phase or DNA replication (Mathews and van Holde, 1990). Since the peaks in OD seen in the term and preterm CBMCs occurred at 4 and 12 hours, these values cannot be attributed to proliferation. It is suggested that an increase in mitochondria or mitochondrial activity may account for the peaks in OD. Phenazine ethosulfate in the MTS reagent, is bioreduced to fomazan by NADH and NADPH, that are produced by mitochondrial dehydrogenase enzymes between the inner and outer membrane of the mitohondrian (Berridge and Tan, 1993). It is known that mitochondria provide ATP for T-cell activation by electron transport and oxidative phosphorylation (Perl *et al.*, 2002). Cellular energy is sustained by the β-oxidation of LCPUFAs in the mitochondria, and such metabolism, is dependent on NADPH (Janssen and Stoffel, 2002). Thus it is speculated, that NADPH activity in the mitochondria of term, preterm and DNI CBMCs in culture media supplemented with AA and DHA, increased in order to maintain β-oxidation of LCPUFAs, and were thus available in higher quantities to bioreduce the MTS reagent, resulting in peaks in OD. EPA has been shown to be preferentially β-oxidised by the mitochondria and DHA and AA by the peroxisomes (Madsen *et al.*, 1998 and Hiltunen *et al.*, 1986). Thus it seems plausible that AA and DHA had an effect of neonate CBMCs in culture even though it was more metabolic than proliferative.

Figures 6.1 to 6.4 showed the peaks seen at 4 hours for term cells and 12 hours for preterm cells were consistent even in cells cultured in unsupplemented (control) medium. FCS, essential in cell culture for the maintenance of cell lines, contains several lipids including triacylglycerols, cholesterol, phospholipids and non-esterified fatty acids (Yacoob *et al.*, 1995) and may have contributed to the increases in metabolic activity in cells cultured in un-supplemented media, as postulated above. This could be further investigated by comparing the effects of FCS with delipidated FCS on CBMCs in culture.

As mentioned previously, workers such as Calder and Newsholme (1992) and Rotondo *et al.* (1996) found that LCPUFAs such as AA and DHA were inhibitory to adult lymphocyte proliferation. Zhao *et al.* (2002) showed that fetal and neonatal T-cells did not proliferate in response PHA stimulation as well as adult T-cells. PHA is a sugar-binding lectin and a well-established T-cell mitogen (Benjamini, 2000), that preferentially stimulates CD4-expressing T-cells (Hickling, 1998). It is used routinely for the activation of human T-cells in culture over a period of 12-72 hours. The ability to respond to PHA *in vitro* is indicative of the functional abilities of the cells.

This study showed term CBMCs cultured over 3 days in medium containing PHA and 256μM AA showed proliferative increases in the CD4 population of leukocytes particularly CD4 T-cells and B-cells (CD19/45), compared to the untreated controls (figure 6.5). It is difficult to interpret whether the results from this study are in complete agreement with Zhao *et al.* (2002), as the control cells in media with PHA would have to be compared to control cells in media without PHA. However, it is clear from the results that term CBMCs respond to PHA stimulation when AA is added to the culture media (figure 6.5). This is given by the enhanced expression of CD25 (IL-2 responsive

cell) expression on T and B-cells as well as higher levels of CD8/45RO and CD8/45RA compared to CBMCs treated with PHA only (figure 6.5).

It is also possible that CD34+ precursor cells (not tested) within the CBMC population cultured with AA-supplemented media, were differentiating and thereby expressing T-helper cell markers on their surfaces. As T and B-cell expression was somewhat enhanced in cells that were cultured with AA plus PHA, it might therefore be inferred that the contribution of AA or AA-derived metabolite-driven differentiation was greater than that of PHA. In support of this hypothesis, the AA-derived leukotriene B4 has been shown to induce differentiation and proliferation of CD34+ stem cells (Chung *et al.*, 2005).

6.5 Conclusion

The results of the preliminary cell viability colorimetric analyses outlined in this chapter, showed that optimal concentrations of AA and DHA (256μM and 244μM respectively) increased optical density over short time periods in term, preterm and DNI CBMCs *in vitro*. This data was not in complete agreement with other workers who showed that AA and DHA were toxic to cells in culture. It was postulated that these increases in optical density at short time periods were due to mitochondrial LCPUFA metabolism as opposed to cellular proliferation. Term T-cells proliferated in culture media containing PHA and AA over 3 days. The data suggests that AA may facilitate the response of T-cells to the mitogen.

The effect of AA and other LCPUFAs need to be tested on preterm and DNI CBMCs in order to gain a more complete understanding of the immunocompetence of these cells and their response to mitogens in culture.

Chapter 7

7.1 Summary Discussion

There is a positive correlation between maternal polyunsaturated fatty acid (PUFA) consumption and neonatal PUFA status (Hornstra, 2000). Placental selection and biomagnification favours the transfer of AA to the fetus which increases from mid-term to term (Crawford *et al.*, 2003). This means that "high risk" neonates such as preterm and DNI neonates with existing immune deficiencies are further subjected to deficits in LCPUFA supply.

DNI neonates have compromised immune systems (Rich *et al.*, 1997 and Clerici *et al.*, 2000), and low plasma levels of EFAs such as LA (Agostoni *et al.*, 1998). As LCPUFAs are directly and indirectly involved in the functioning of the immune system, and since the impact of the loss of LCPUFAs on the neonatal immune system had not previously been assessed, it was of importance to look at the fatty acid status of the CBMC membranes of term, preterm and DNI neonates as well as their immune cell subsets with relation to gestational age.

This study has raised 4 important findings:

- Significantly lower levels of CD4 and CD8 subsets in preterm and CD4 subsets in DNI CBMCs when compared to term;
- The dominance of AA over other LCPUFAS in the PC and PE fraction of CBMCs of all neonates;
- Lower levels of AA and other LCPUFAs in preterm and DNI CBMC membranes when compared to term, and;
- Statistically significant correlations between cord blood lymphocyte subset levels and fatty acids.

Lower levels of immune cell subsets in preterm neonates may be attributed to low gestational age as the levels of CD4 and CD8 T-cells are significantly positively correlated with gestational age (chapter 3). Other workers have shown previously that preterm neonates have fewer T-lymphocytes, particularly T-helper cells (Wilson *et al.*, 1985 and Juretić *et al.*, 2001) compared to term neonates, which may account for their poor immune response to infection. It has also been shown that preterm cord blood contained higher levels of CD34+ haematopoietic stem cells than term cord blood, and that these cells were committed for erythroid rather than myeloid cells at low gestational ages (Meister *et al.*, 1994). The latter may account for the low levels of CD4 and CD8 immune cell subsets seen in preterm neonate cord blood compared to term.

Lower levels of CD4 subsets (chapter 3) and consequently lower CD4:CD8 ratio was reported in HIV-infection as well as in seroreverters (Clerici *et al.*, 2000). In DNI neonates the lower levels of CD4 cells and higher levels of CD4 memory cells have been shown to be as a result of exposure of the fetus to viral particles Clerici *et al.*, 2000). In this study, lower numbers of CD4 memory cells seen in this study seem to preclude intrauterine exposure to viral particles. However, definitive proof of this would be to ascertain the HIV CD4-T-cell function of these cells, which would be raised if exposed to viral particles as shown in experiments by Nielson *et al.*, 2001. Impairment in the function of progenitor cells (Nielson *et al.*, 2001) or increased T-cell susceptibility to apoptosis (Economides *et al.*, 1998) may also account for the low levels of CD4 T-cells seen in DNI cord blood compared to term.

HIV-infected patients are deficient in plasma omega 3 and omega 6 fatty acids (Begin *et al.*, 1988 and 1989) and have lower omega 6 LCPUFA levels (Peck *et al.*, 1993). Thus the placental provision of LCPUFAs would be adversely affected in HIV-positive mothers. This is borne out by the fact that HIV-infected children, have low levels of plasma LA and higher levels of AA indicated increased LCPUFA turnover (Agostoni *et al.*, 2000).

AA accounted for 34% of the PE fraction and 15% of the PC fraction in the mononuclear cell membranes of term neonates (chapter 4), far exceeding that of the other omega 6 or omega 3 fatty acids. As AA only occupies the sn-2 position in the phosphoglycerides, this means that every third of the principle polar phosphoglyceride contained AA.

In adult human monocytes, AA has been reported to account for approximately 20% of the total membrane phospholipids (Stossel *et al*, 1974 and Marinetti and Cattieu, 1982) with the CD4+ T cell fraction being approximately 25% (Klein *et al.*, 1992) and monocyte and macrophage fraction approximately 20-25% (Laganiere and Fernandes, 1991 and Scott *et al.*, 1980). However these findings cannot be directly compared with the results presented in this thesis as they represent percentages of the total membrane LCPUFAS and do not differentiate between the PC and PE membrane phosphoglycerides.

Omega 3 fatty acids, which are important modulators of immune function (Tomobe *et al.*, 2000, Calder *et al.*, 2002 and Mori and Beilin, 2004), represented less than a tenth of the amount of AA in CBMC membranes. EPA accounted for less than 0.5% and DHA, for approximately 2% in the PC and only 5% of the PE polar phosphoglycerides.

This preponderance of AA and paucity of omega 3 fatty acids is an exaggeration of the placental biomagnification that preferentially selects AA to any other super-unsaturated fatty acid for transfer to the fetus, with a reduction in precursors such as linoleic acid and eicosapentaenoic acid (Crawford *et al.*, 2003). The preferential selection of AA and reduction in omega 3 fatty acids from the circulating phosphoglyceride pool in the fetus by lymphocytes, suggests that prenatally, there may be further selective biomagnification in favour of AA by the maturing lymphocyte. It has been shown that in the lymphocytes of rats and rabbits there is preferential incorporation of AA and LA (Calder, 1994 and Rode *et al.*, 1982), although when exogenous DHA was made available to the lymphocytes of healthy adult volunteers, it was incorporated at the expense of AA (Endres *et al.*, 1989).

The findings presented here in respect to AA are complemented by an earlier study on the serum phospholipid fatty acid spectra of healthy term neonates showing that AA was the most predominant polyunsaturated fatty acid, and that its levels in neonatal serum phospholipids was significantly higher ($p<0.001$) than that in adults (Hardell and Walldius, 1980).

Other studies have shown positive correlations ($p<0.01$) for AA and birth weight in plasma choline phosphoglycerides (Leaf *et al.*, 1992) and plasma triglycerides ($p=0.01$) (Koletzko and Braun, 1991). Leaf *et al.*, 1992 showed that DHA was correlated with gestational age, which implies that preterm neonates are born at a time when their need for LCPUFAs such as DHA and AA are greatest for growth and development. Statistically significant correlations for AA and birthweight and DHA and gestational age in CBMCs are also shown in this study (chapter 4).

Despite the high levels of AA seen in the preterm CBMCs, transplacental gradients suggest that during periods of high demand preterm babies may have a deficiency of AA and DHA (Crawford *et al.*, 2003). As this was observed in the analysis of preterm CBMCs in this study, it may imply that there would be a cost to cell functioning due to the low level of a fatty acid that is intrinsic to inflammatory interactions and the production of eicosanoids. However this composition only reflects the status of the PC and PE phosphoglycerides, and further assessments of other polar phosphoglycerides are required.

DNI CBMCs showed very similar levels of AA and DHA to term. However in these cells the level of LA was higher than term CBMCs. HIV-positive women have high fatty acid and cell turnover and consequently the DNI neonate plasma levels of AA and LA are low (Agostoni *et al.*, 2000). The high levels of AA and LA seen in this study suggests that these LCPUFAs may be preferentially incorporated into the mononuclear cells of cord blood.

Mead acid is used as a marker of EFA deficiency as low Mead acid to AA levels suggests a deficiency in LA. Delta 6 and delta 5 desaturases (figure 1.3) convert LA and ALA to AA and DHA respectively. In the absence of LA and ALA, oleic acid is converted to Mead acid. The preterm Mead acid level suggested that preterm CBMCs may not have been EFA deficient however the Mead acid to AA ratio was lower than healthy term samples. Although delta 6- and delta 5-desaturase activities were observed in liver microsomes of human neonates, they were lower than previously reported in adult humans and other mammals (Poisson *et al.*, 1993). It is therefore plausible that there would be even less desaturase activity at lower gestational ages accounting for the lower Mead acid levels.

The Mead acid to AA ratio in DNI neonates suggested that there DNI CBMCs suffered a deficit in EFAs. This was invalidated by the high levels of LA in the DNI CBMCs. Studies have shown that the cord blood and peripheral blood leukocytes of children born to HIV-positive were at risk for mitochondrial damage (Poirier, *et al.*, 2003). This was particularly true of those exposed to AZT in utero. It is therefore plausible that damage to other cellular organelles and dysregulation of the micosomal desaturase may have occurred during gestation. Since delta 5 and delta 6 desaturase activity in the microsomes are responsible for the conversion of LA to AA and oleic acid to Mead acid, disruptions in this mechanism might be altered by damage to the microsomes. Therefore the high Mead acid and simultaneously high LA levels found in DNI CBMCs may be inconclusive for EFA deficiency.

Surprisingly, whilst AA is present in such high quantities in the PE fraction (chapter 4), it is not correlated with the immune subset levels (chapter 5). It may be postulated from this that AA does not have a specific function in the PE fraction, but a more generic one, for example contributing to the curvature of the membrane. It is known that differences in membrane curvature and cholesterol content alter the array of PE molecules on the surfaces of membrane bilayers and that this could have profound effects on a number of critical membrane functions and processes (Williams *et al.*, 2000). Previous work on cultured neonatal rat cardiomyocytes indicated that the cytoplasmic (inner) leaflet is more unsaturated than the outer one, and that the phospholipids predominantly located in the inner leaflet including PE were rich in stearic acid (C18:0) and AA (Matos *et al.*, 1990).

However, data on placental transfer (Crawford, 2000) and the extraordinarily high concentration of AA in the lymphocytes of term newborns (chapter 4) as well as the correlation between AA in the PC fraction and CD4 and CD8 T-cells (chapter 5) suggests its importance in the development of the immune system along with the development of vascular tissues.

Activated T-cells and leukocytes were significantly positively correlated with docosatetraenoic acid (DTA), LA and AA. Mead acid was significantly negatively correlated with activated T- and B lymphocytes and leukocytes indicating that EFAs and LCPUFAs are preferentially incorporated into the membranes of cells that are undergoing proliferation. The preferential incorporation of LCPUFAs into the cell membranes of lymphocytes and IL-2 responsive or activated cells indicates the importance of these fatty acids in the functioning and protection of the immune cells. Kohn *et al.*, 1980 showed that unsaturated free fatty acids (such as LA and AA in culture) reduced the infectivity of enveloped viruses at concentrations of 5-25μg/ml.

Since a significant deficit of several essential LCPUFAs was found in the membranes of preterm and DNI CBMCs, preliminary studies were undertaken investigate the effect of *in vitro* supplementation with AA and DHA on the viability and proliferation of CBMCs. Other workers have reported that LCPUFAs are toxic to lymphocytes in culture often at very small concentrations (Calder and Newsholme, 1992, Rotondo *et al.*, 1996 Pompeia *et al.*, 2002 and Lima *et al.*, 2002). This study did not show AA and DHA toxicity to CBMCs over 24 hours. Instead it was postulated that the peaks in optical density noticed at optimal concentrations of AA and DHA at short time periods was as a result of mitochondrial metabolism for ATP production (see chapter 6).

Zhao *et al.*, 2002 reported that fetal and neonatal T-cells did not respond to PHA in culture. This study showed that in the presence of AA, proliferation in T-cells did occur. Since proliferation requires IL-2 receptor expression which is correlated with the production of IL-2 (Karsten *et al.*, 1994) this may imply that AA facilitates the expression of the IL-2 receptor and thereby facilitates proliferation. This is supported by findings in a study on unstimulated lymphocytes from the thymus of calves and rabbits, Goppelt *et al.*, (1985) which showed that exogenous LCPUFAs were preferentially incorporated and esterified to phospholipids and triacylglycerols, and from triacylglycerols to phospholipids. AA was preferentially transferred to PE and phosphatidylinositol (Goppelt *et al.*, 1985), which was enhanced after the cells were stimulated with a mitogen.

It was therefore regrettable that similar studies could not be undertaken with preterm CBMCs as due to the higher levels of activated cells seen in preterm cord blood, there may have been an greater proliferative response compared to term CBMCs.

7.2 Summary Conclusion

This study has established that preterm and DNI neonates have lower levels of immune cell subsets compared to term neonates. In addition to this the cord blood mononuclear cells of preterm and DNI neonates have lower levels of LCPUFAs. The level of LCPUFAs in neonatal cell membranes was significantly correlated with immune cell subset levels. These findings taken together may contribute to what is currently known about the impact of poor maternal diet or placental insufficiency of LCPUFAs on the development and maturation of the fetal immune system.

The results presented in this study may also contribute to ultimately supplementing women in antenatal clinics with the aim of improving pregnancy outcomes. In the case of HIV-infected mothers, who are themselves LCPUFA deficient, dietary supplementation may benefit nutritional status of both the mother and her child. Alternatively supplementation may even modulate HIV infectivity and its resultant inflammatory disorders. This would be particularly useful in developing countries that carry approximately 90% of the global HIV-1 burden, where the healthcare infrastructure is poor, antiretroviral drugs are a premium, and malnutrition is rife.

Appendix 1

A list of constituents of the RPMI 1640 (1X)

Component	g/L	Component	g/L
INORGANIC SALTS		L-Serine	0.03
$Ca(NO_3)_2 \bullet 4H_2O$	0.1	L-Threonine	0.02
$MgSO_4$ (anhyd)	0.04884	L-Tryptophan	0.005
KCL	0.4	L-Tyrosine•2Na•$2H_2O$	0.02883
$NaHCO_3$	2.0	L-Valine	0.02
NaCl	6.0	**VITAMINS**	
Na_2HPO_4 (anhyd)	0.8	D-Biotin	0.0002
AMINO ACIDS		Choline Chloride	0.003
L-Arginine (free base)	0.2	Folic Acid	0.001
L-Asparagine (anhyd)	0.05	myo-Inositol	0.035
L-Aspartic acid	0.02	Niacinamide	0.001
L-Cystine•2HCL	0.0652	p-Amino benzoic acid	0.001
L-Glutamic acid	0.02	D-Pantothenic acid•½Ca	0.00025
L-Glutamine	0.3	Pyridoxine•HCL	0.001
Glycine	0.01	Riboflavin	0.0002
L-Histidine (free base)	0.015	Thiamine•HCL	0.001
Hydroxy-L-Proline	0.02	Vitamin B-12	0.000005
L-Isoleucine	0.05	**OTHER**	
L-Leucine	0.05	D-Glucose	2.0
L-Lysine•HCL	0.04	Glutathione (reduced)	0.001
L-Methionine	0.015	Phenol Red•Na	0.0053
L-Phenylalanine	0.015	**HEPES**	4.77
L-Proline	0.02		

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