Characterisation of Polyunsaturated Fatty Acid Synthesis
in Peripheral Blood Mononuclear Cells

By

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Declaration

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service TurnitinUK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.

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Summary

Conversion of the essential n-3 (18:3n-3) and n-6 (18:2n-6) fatty acids to longer chain polyunsaturated fatty acids (PUFA) involves sequential desaturation and elongation reactions. Previous studies have reported gender differences in n-3 PUFA synthesis, whereas the effect of age is less clear. n-3 PUFAs are reported to have important effects on immune cell function. A previous study reported long chain PUFA synthesis in mitogen stimulated but not quiescent peripheral blood mononuclear cells (PBMCs). However, the underlying mechanism is not known.

PUFA synthesis was investigated in PBMCs incubated with [1-13C]18:3n-3 for 48 h. Activation with the T-lymphocyte mitogen concanavalin A (Con A) increased PUFA synthesis. 22:6n-3 synthesis was not detected. [1-13C] incorporation was greatest for 20:3n-3 suggesting initial chain elongation is an important fate for 18:3n-3. Con A increased expression of three key genes (FADS2, FADS1 and ELOVL5) involved in PUFA synthesis, suggesting upregulation of the pathway is controlled at the transcriptional level. ELOVL2 expression was negligible, possibly explaining the lack of 22:6n-3 synthesis. Con A increased methylation of 12 CpGs in the FADS2 promoter contradicting the general view that DNA methylation represses transcription. Subsequent 5’RACE analysis verified that activated PBMCs were not using an alternative promoter for FADS2 transcription. Contrary to expectation, 18:3n-3 conversion in activated PBMCs was not affected by gender or menopausal status and there was no clear age effect.

PUFA synthesis was constitutive in the Jurkat T-lymphocyte leukaemic cell-line and was higher than in PBMCs. FADS2, FADS1 and ELOVL5 mRNA expression was also higher in Jurkat cells and was associated with 50% lower methylation of 17 CpGs in the FADS2 promoter, suggesting transcriptional dysregulation of PUFA synthesis in Jurkat cells involves altered DNA methylation.

These findings have provided novel insights into the regulation of PUFA biosynthesis in PBMCs and upregulation of the pathway in activated PBMCs suggests that newly synthesised PUFAs may be important for cell function.
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<td>ACC</td>
<td>Acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AP</td>
<td>Atom percent</td>
</tr>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>APE</td>
<td>Atom percent excess</td>
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<td>ATP</td>
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<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol esters</td>
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<tr>
<td>CEDAR</td>
<td>Centre for Endocrinology and Diabetic Research</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf intestine alkaline phosphatase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>D6KO</td>
<td>Delta-6 desaturase knockout</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
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<td>DNA methyltransferase</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>EAT</td>
<td>Ehrlich ascites tumour</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Authenticated Cell Cultures</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ELOVL</td>
<td>Elongation of very long fatty acids</td>
</tr>
<tr>
<td>FADS1/FADS2</td>
<td>Fatty acid desaturase 1/2</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detection</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
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<td>Fluorescence channel 1/2</td>
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<td>Forward scatter</td>
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<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-C-IRMS</td>
<td>Gas chromatography-combustion-isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GPCR</td>
<td>G coupled protein receptor</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HDACs</td>
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<td>High density lipoprotein</td>
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<td>HMTs</td>
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<td>Immunoglobulin G</td>
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<td>Interleukin</td>
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<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<td>LOX</td>
<td>Lipoxygenase</td>
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<td>Leukotriene</td>
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<tr>
<td>LX</td>
<td>Lipoxin</td>
</tr>
<tr>
<td>MeCP2</td>
<td>Methyl cytosine binding protein 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Maloney Murine Leukaemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ncRNA</td>
<td>Non-coding RNA</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NRES</td>
<td>NHS Research Ethics Service</td>
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<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Pee Dee Belemnite</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
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<td>piRNA</td>
<td>Piwi-interacting RNA</td>
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</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PRC2</td>
<td>Polycomb repressive complex 2</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>REC</td>
<td>Research Ethics Committee</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean squared</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RPE</td>
<td>R. Phycoerythin</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>RSCH</td>
<td>Royal Surrey County Hospital</td>
</tr>
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<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
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<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
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<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase 1</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SPM</td>
<td>Specialised pro-resolving mediator</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory binding protein</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TAP</td>
<td>Tobacco acid pyrophosphatase</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
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<tr>
<td>TX</td>
<td>Thromboxane</td>
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### Fatty acid nomenclature

<table>
<thead>
<tr>
<th>Notation</th>
<th>Trivial Name</th>
<th>Systematic name</th>
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<tbody>
<tr>
<td>18:3n-3</td>
<td>α-Linolenic acid</td>
<td>all-cis-9,12,15-Octadecatrienoic acid</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>Stearidonic acid</td>
<td>all-cis-6,9,12,15-Octadecatetraenoic acid</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>Eicosatrienoic acid</td>
<td>all-cis-11,14,17-Eicosatrienoic acid</td>
</tr>
<tr>
<td>20:4n-3</td>
<td>Eicosatetraenoic acid</td>
<td>all-cis-8,11,14,17-Eicosatetraenoic acid</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>Eicosapentaenoic acid</td>
<td>all-cis-5,8,11,14,17-Eicosapentaenoic acid</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>Docosapentaenoic acid</td>
<td>all-cis-7,10,13,16,19-Docosapentaenoic acid</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>Docosahexaenoic acid</td>
<td>all-cis-4,7,10,13,16,19-Docosahexaenoic acid</td>
</tr>
<tr>
<td>24:5n-3</td>
<td>Tetracosapentaenoic acid</td>
<td>all-cis-9,12,15,18,21-Tetracosapentaenoic acid</td>
</tr>
<tr>
<td>24:6n-3</td>
<td>Tetracosahexaenoic acid</td>
<td>all-cis-6,9,12,15,18,21-Tetracosahexaenoic acid</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>Linoleic acid</td>
<td>all-cis-9,12-Octadecadienoic acid</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>γ-Linolenic acid</td>
<td>all-cis-6,9,12-Octadecatrienoic acid</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>Eicosadienoic acid</td>
<td>all-cis-11,14-Eicosadienoic acid</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>Dihomo-γ-linolenic acid</td>
<td>all-cis-8,11,14-Eicosatrienoic acid</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>Arachidonic acid</td>
<td>all-cis-5,8,11,14-Eicosatetraenoic acid</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>Adrenic acid</td>
<td>all-cis-7,10,13,16-Docosatetraenoic acid</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>Docosapentaenoic acid</td>
<td>all-cis-4,7,10,13,16-Docosapentaenoic acid</td>
</tr>
<tr>
<td>16:0</td>
<td>Palmitic acid</td>
<td>Hexadecanoic acid</td>
</tr>
<tr>
<td>17:0</td>
<td>Margaric acid</td>
<td>Heptadecanoic acid</td>
</tr>
<tr>
<td>18:0</td>
<td>Stearic acid</td>
<td>Octadecanoic acid</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>Palmitoleic acid</td>
<td>cis-9-Hexadecenoic acid</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>Oleic acid</td>
<td>cis-9-Octadecanoic acid</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1 Fatty acid structure

Fatty acids consist of a hydrocarbon (acyl) chain with a carboxyl group at one end and a methyl group at the other end. The acidic carboxyl group reacts readily to form ester links with hydroxyl groups. Fatty acids can be esterified to produce more complex lipids such as cholesterol esters (CE) and acylglycerols, which include triacylglycerols (TG) and phospholipids. Fatty acids are also present in the body as non-esterified fatty acids (NEFA) where they are bound to serum albumin for transport to target tissues. Phospholipids are the main constituent of biological membranes. Common phospholipids found in membranes include the phosphoglycerides: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), diphosphatidylglycerol and phosphatidylinositol. Sphingomyelin is another class of phospholipid found in membranes but in contrast to the phosphoglycerides, sphingomyelin is not derived from glycerol.

Long chain fatty acids (>12 carbons) are most common in mammalian plasma and tissues. They can be classified as saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids based on the number of double bonds they contain—zero, one or two or more, respectively. Double bonds can be in either the cis or trans configuration. The length of the acyl chain and the number, position and orientation of the double bonds, all influence the physical and biological properties of the fatty acid.

1.2 Fatty acid nomenclature

Fatty acids can be named using a trivial name, systematic name, or shorthand notation. The traditional systematic name denotes the number of carbons in the acyl chain and, when present, the position and configuration of double bonds relative to the carboxyl carbon (carbon 1). Therefore, using this naming system, the 18 carbon saturated fatty acid stearic acid (trivial name) is written as octadecanoic acid and the 18 carbon PUFA α-linolenic acid (trivial name) is written as cis 9, cis 12, cis 15-octadecatrienoic acid or more simply, all-cis-9,12,15-octadecatrienoic. Shorthand notation is now regularly used for naming fatty acids and is based on the fatty acid chain length and the number and position of double bonds. This naming system lists the first double bond counting from the methyl carbon of the fatty acid, which is sometimes referred to as the omega (ω) carbon. For example, α-linolenic acid is written as 18:3n-3. This means it has a chain length of 18 carbons (18), contains three double bonds (:3) and the first double bond occurs on the third carbon counting from the methyl end (n-3) of the fatty acid. The shorthand notation for stearic acid is 18:0 as it consists of 18 carbons and no double bonds. The shorthand notation for naming fatty acids is useful when considering fatty acid biosynthesis as they are are elongated from their carboxyl end and therefore fatty acids formed in the same biosynthetic pathway have the same ‘n’ notation. For...
example, elongation of 18:3n-3 and linoleic acid (18:2n-6) gives rise to the n-3 and n-6 series of PUFA, respectively. Therefore, the shorthand notation will be used in this thesis.

1.3 **Biosynthesis of fatty acids**

Fatty acid synthesis (lipogenesis) involves successive addition of two carbon units from acetyl-CoA to the fatty acid chain. Glucose breakdown via glycolysis results in the production of pyruvate, which is converted to acetyl-CoA by oxidative decarboxylation (3). Synthesis of fatty acids from acetyl-CoA requires formation of the activated intermediate malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) and then two carbon units from malonyl-CoA are added to the activated carboxyl end of the growing acyl chain by fatty acid synthase (FAS). The primary product of de novo synthesis is palmitic acid (16:0) but the action of thioesterases can also result in the release of shorter chain fatty acids (2).

1.4 **Fatty acid modification**

Newly synthesised fatty acids and fatty acids obtained from the diet can be further modified by elongation and desaturation or by hydroxylation; the latter has been demonstrated in the nervous system for the formation of some myelin lipids (2). 16:0 can be converted to 18:0 by the action of elongase enzymes. 16:0 or 18:0 can be converted to 16:1n-7 and oleic acid (18:1n-9), respectively, by the enzyme delta-9 desaturase, also referred to as stearoyl-CoA desaturase, which catalyses the addition of a cis double bond between carbon 9 and 10 relative to the carboxyl end of the fatty acid. In mammals, desaturation and elongation of fatty acids occurs in the endoplasmic reticulum (3).

Important substrates for the synthesis of PUFAs are the essential fatty acids 18:2n-6 and 18:3n-3, which can only be obtained from the diet. This is because mammals do not have the enzymes that introduce double bonds beyond carbon 9 in the acyl chain and consequently cannot synthesise 18:2n-6 and 18:3n-3. These fatty acids can be obtained from the diet by eating higher plants that have the enzymes to insert new double bonds between existing double bonds and the terminal methyl group. Plants are able to desaturate 18:1n-9 to yield 18:2n-6 (catalysed by delta-12 desaturase), which can then be further desaturated by delta-15 desaturase to produce 18:3n-3 (1). These fatty acids are the simplest of the n-6 and n-3 series of fatty acids and although they cannot be synthesised by mammalian cells they can undergo further desaturation and elongation.
1.4.1 Desaturation and elongation of n-3 and n-6 PUFA

Both n-3 and n-6 PUFA are metabolised by the same series of enzymes (Figure 1.1). This pathway was characterised by a number of experiments in rat liver and has been comprehensively reviewed (4). The initial reaction, catalysed by delta-6 desaturase, is considered the rate limiting reaction of the pathway. Since both n-3 and n-6 PUFA are metabolised by the same series of enzymes there is potential for competition. The affinity of delta-6 desaturase is greater for 18:3n-3 than for 18:2n-6 (4) but because 18:2n-6 is more common than 18:3n-3 in most human diets (5) the conversion of n-6 PUFA is greater (6).

The reactions occur in the endoplasmic reticulum, except the final reaction in the pathway which results in the formation of docosahexaenoic acid (22:6n-3) and n-6 docosapentaenoic acid (22:5n-6). With respect to n-3 PUFA, the initial reaction involves the conversion of 18:3n-3 to stearidonic acid (18:4n-3) by delta-6 desaturase. Next, addition of two carbons by elongase-5 activity results in the formation of eicosatetraenoic acid (20:4n-3). Delta-5 desaturase catalyses the conversion to eicosapentaenoic acid (20:5n-3) and subsequent addition of two carbons by elongase activity yields docosapentaenoic acid (22:5n-3). Conversion of 22:5n-3 to docosahexaenoic acid (22:6n-3) is generally accepted to occur by the following pathway: elongation of 22:5n-3 to tetracosaheptaenoic acid (24:7n-3) by elongase-2, followed by delta-6 desaturation of 24:5n-3 to form tetracosapentauenoic acid (24:6n-3), which is then translocated from the ER to the peroxisome where one cycle of the β-oxidation pathway shortens the chain by two carbons to form 22:6n-3. How the translocation steps and β-oxidation are regulated is not entirely known but it could represent a point of metabolic control that results in regulation of 22:6n-3 synthesis independently from the synthesis of the preceding long chain n-3 PUFA in the pathway (4).

The conversion of 22:5n-3 to 22:6n-3 has been a matter of debate and an alternative mechanism, whereby 22:5n-3 is converted to 22:6n-3 by delta-4 desaturase activity without peroxisomal involvement, has been suggested (7). However, stable isotope experiments in rat liver microsomes and hepatocytes and human Y-79 retinoblastoma cells have demonstrated that synthesis of 22:6n-3 occurs independently of delta-4 desaturation by the pathway detailed above (8, 9). Furthermore, in human cells, specific inhibition of delta-6 desaturase by 2,2-diphenyl-5-(4-[[1 E]-pyridin-3-ylmethylidene]amino)piperazin-1-yl)pentanenitrile (SC-26196) reduced conversion of stable isotope labelled 22:5n-3 and 24:5n-3 to 22:6n-3 by 75% and 84%, respectively, strongly supporting that 22:6n-3 synthesis involves delta-6 desaturation of 24:5n-3 (10). However, in human cells, the delta-6 desaturase enzyme was recently demonstrated to have delta-4 desaturase activity and the ability to directly catalyse delta-4 desaturation of 22:5n-3 to yield 22:6n-3 (11). This could explain the reduced conversion of 22:5n-3 to 22:6n-3 in the presence of SC-26196 shown by Harmon et al.,
2003, if delta-6 desaturase catalysed delta-4 desaturation of 22:5n-3 to form 22:6n-3 (10). However, the reduced conversion of 24:5n-3 to 22:6n-3 in the presence of SC-26196 indicated that the pathway involving delta-6 desaturation to form 22:6n-3 was functioning (10).

![Figure 1.1. The n-3 and n-6 PUFA desaturation and elongation pathway.](image)

The enzymes that catalyse each conversion are in the centre with their corresponding gene written above. Delta is represented by the symbol Δ.

### 1.4.2 Delta-6 and delta-5 desaturase enzymes

Desaturase enzymes introduce cis double bonds between particular carbons of the fatty acid chain. Delta-6 and delta-5 desaturases introduce a double bond after the sixth and fifth carbon atoms, respectively, from the carboxyl end of the fatty acid carbon chain. They are classified as front-end desaturases as they are able to insert a double bond between the pre-existing double bond and the carboxyl end of the fatty acid. In eukaryotic cells, delta-6 and delta-5 desaturases are anchored in the ER membrane. They contain an N-terminal cytochrome b5-like domain and a C-terminal desaturation domain (12). Three conserved histidine-rich motifs act at the di-iron catalytic centre of the desaturases (13). Cytochrome b5 functions in the electron transfer system in the desaturation reaction and mutation or deletion of the cytochrome b5-like domain have demonstrated it is essential for the activity of rat delta-6 desaturase, which could not be rescued by endogenous cytochrome b5 (14).
In humans, delta-6 desaturase is encoded by the *FADS2* gene and delta-5 desaturase is encoded by the *FADS1* gene. Delta-6 and delta-5 desaturase enzymes are expressed in many human tissues including the lung, kidney, pancreas and skeletal muscle, with the greatest activities found in the liver, heart and brain (15, 16). They are clustered within a 92 kb region on chromosome 11 at 11q12-q13.1 with a third *FADS* gene (*FADS3*) and their similar exon/intron organisation suggests evolution from a common ancestor by gene duplication events (17). *FADS1* and *FADS2* are positioned in head-to-head orientation with an 11 kb region between the first exons of both genes. It has been suggested that, due to the proximity of the *FADS1* and *FADS2* promoters, the 11 kb intergenic region might contain common regulatory sequences that coordinate the transcription of both genes (16, 18).

The *FADS2* gene product displays both diverse substrate specificity and desaturase activities (11, 19). It is reported to act on at least seven substrates including 18:2n-6, 18:3n-3, eicosadienoic acid (20:2n-6), 20:3n-3, 24:4n-6, 24:5n-3 and 16:0 (20-22) and is capable of introducing double bonds at the delta-6, delta-4 and delta-8 positions (11, 19). Whether the same delta-6 desaturase enzyme catalyses both the desaturation of 18:3n-3 and 24:5n-3 has been a matter for debate. The idea that there may be a distinctive delta-6 desaturase showing selectivity for 24 carbon PUFAs came from observations that most cells exhibit delta-6 desaturase activities and the ability to retro-convert fatty acids yet few cells have the ability to synthesise significant amounts of 22:6n-3 (9). This idea is supported by the existence of different delta-6 desaturase isoforms and experiments in cultured human cells that showed an increase delta-6 desaturase activity on 18:3n-3 under certain conditions but unchanged or decreased 22:6n-3 synthesis (9). However, this observation might be explained by other factors such as the preferential delta-6 desaturation of 18:3n-3 compared with 24:5n-3, shown by competition studies (23) or differential regulation of the elongation or translocation steps involved in synthesis of 22:6n-3. Furthermore, in cloning experiments the same rat delta-6 desaturase has been shown to catalyse both the conversion of 18:3n-3 to 18:4n-3 and the conversion 24:5n-3 to 22:6n-3 (20).

### 1.4.3 Elongase-5 and elongase-2 enzymes

Elongase enzymes function in extending the acyl chain of long chain fatty acids; an ER-bound process involving four enzymes and fatty acyl CoA, malonyl CoA and NADPH as substrates, allowing addition of two carbons to the fatty acyl chain per cycle (24). The initial and rate-limiting condensation reaction is catalysed by elongase enzymes (25). Elongase enzymes are encoded by the *ELOVL* (elongation of very long chain fatty acid) genes. Proteins encoded by *ELOVL1, ELOVL3, ELOVL6* and *ELOVL7* are selective for saturated and monounsaturated fatty acids and *ELOVL2, ELOVL4* and *ELOVL5* encoded proteins prefer PUFAs as substrates (25). The elongase-5 and
elongase-2 enzymes are encoded by the *ELOVL5* and *ELOVL2* genes, respectively, and are responsible for elongation of n-3 and n-6 PUFAs. Both genes are located on chromosome 6 with *ELOVL5* at 6p12.1 and *ELOVL2* at 6p24.2. *ELOVL5* is widely expressed with highest levels reported in the adrenal glands and testes (26) whereas *ELOVL2* displays more tissue specific expression, being found in tissues rich in 22:6n-3, such as the liver, testis and brain (27, 28). There has been some uncertainty as to where Elongase-5 and Elongase-2 function in the PUFA synthesis pathway and they display some overlap in their functions. Elongase-5 is involved in the elongation of 18 and 20 carbon PUFAs, while elongase-2 utilises 20 and 22 (to produce 24 carbon precursors for formation of 22:6n-3 and 22:5n-6) carbon PUFAs as substrates (26, 29-31). *ELOVL5* has been shown to be expressed at much higher levels than *ELOVL2* in rat liver and heart indicating that it might be more important for the conversion of 20:5n-3 and 20:4n-6 (29). However, 20:5n-3 elongation was maintained in the liver of *ELOVL5* knockout mice and interestingly *ELOVL2* was upregulated in these mice compared with wild-type (WT) controls (32). This suggests that elongase-2 might be important for 20 carbon elongation when elongase-5 is diminished although it is not clear whether one or both these enzymes normally contribute to the conversion of 20:5n-3 and 20:4n-6 (29).

1.4.4 The alternative delta-8 desaturation pathway for n-3 and n-6 PUFA synthesis

Figure 1.2 shows an alternative pathway for n-3 and n-6 PUFA synthesis consisting of elongation of 18:3n-3 to eicosatrienoic acid (20:3n-3) and 18:2n-6 to 20:2n-6. This is followed by delta-8 desaturation of 20:3n-3 and 20:2n-6 to form 20:4n-3 and 20:3n-6, respectively, products in the conventional pathway (19, 33). The operation of this pathway has been verified by molecular cloning and functional characterisation experiments in unicellular organisms (34, 35). However, the occurrence and importance of delta-8 desaturation in mammalian tissues has been a debateable issue. (33, 36, 37). Evidence for delta-8 desaturation has been provided from experiments in rat (38) and human testes (39). Contradictory to these findings, some experiments in mice liver and rat liver and testes have reported delta-8 desaturation as either not occurring or as a questionable route for the biosynthesis of PUFAs. An early study found no evidence for delta-8 desaturation in rat liver in both in vitro and in vivo experiments (37). Another study found no definitive evidence for delta-8 desaturation in rat liver and testes based on measurement of radiolabelled products (36). However, inconclusive mass spectrometry data suggested a small amount of 20:2n-6 may have been metabolised by delta-8 desaturation (36). Analysis of liver lipids in mice fed deuterated 20:3n-3 revealed that only a minor percentage (4.4%) of identified products were formed by delta-8 desaturation (33). A much larger proportion of products were formed by initial delta-5 desaturation of 20:3n-3 to form the unusual fatty acid 20:4(5,11,14,17) and further elongation to 22:4(7,13,6,19). The position of the double bonds relative to the carboxyl carbon have been given here as
20:4(5,11,14,17) has the same shorthand notation as eicosatraenoic acid (20:4n-3). A number of other studies in rat liver and testes and cultured rat, mouse and human cell lines, have reported metabolism of 20:2n-6 and 20:3n-3 to their respective products, 20:3(5,11,14) and 20:4(5,11,14,17), by delta-5 desaturation (36, 37, 40-44).

There is no molecular evidence for a specific gene that encodes delta-8 desaturase activity in mammalian cells. A study in *Saccharomyces cerevisiae* transformed with mammalian FADS2 demonstrated that the delta-6 desaturase enzyme encoded by FADS2 had the ability to catalyse delta-8 desaturation of 20:3n-3 and 20:2n-6 to yield 20:4n-3 and 20:3n-6, respectively (19). However, this was an artificial system where endogenous PUFA synthesis capacity was lacking and therefore important regulatory factors may have been missing. Equivalent results were reported in human breast cancer MCF7 cells which lack delta-6 desaturase activity but do possess delta-5 desaturase activity (43). FADS2 transfected cells were able to convert 20:3n-3 and 20:2n-6 to 20:4n-3 and 20:3n-6, respectively, by delta-8 desaturation (43). The elongase enzyme responsible for the initial reaction in the alternative delta-8 desaturation pathway (the elongation of 18:3n-3 and 18:2n-6 to 20:3n-3 and 20:2n-6, respectively), has not been clearly identified. However, heterologous expression of human *ELOVL5* in *S. cerevisiae* demonstrated that the *ELOVL5* gene product was capable of elongating 18:3n-3 to 20:3n-3, indicating that elongase-5 might be the enzyme catalysing the elongation step in the alternative pathway (26).

![Figure 1.2. The alternative delta-8 desaturation pathway for n-3 and n-6 PUFA synthesis.](image)
1.5 n-3 Polyunsaturated fatty acids

1.5.1 Dietary sources of n-3 PUFAs

The main dietary sources of 18:3n-3 include green leaves, nuts (e.g. walnuts), some seeds (e.g. linseeds) and cooking oils such as soybean oil, rapeseed oil and linseed oil. In the UK, daily intake of 18:3n-3 is estimated to be 1.7 g for males and 1.2 g for females (1). Intake of the n-6 PUFA 18:2n-6 is considerably higher at 13.5 g per day for males and 9.3 g per day for females (1). The longer chain n-3 PUFAs 20:5n-3, 22:5n-3 and 22:6n-3 are consumed in lower amounts compared with 18:3n-3 (45) and are mainly obtained by eating fish, particularly oily fish (e.g. mackerel, herring, salmon, trout and tuna). There is a large variation in fish consumption between individuals (6) but for UK adults consumption of oily fish is estimated to be approximately 50 g/week (46, 47). A proposed recommended daily intake for 18:3n-3 by the European Food Safety Authority is 2 g/day and for 20:5n-3 and 22:6n-3 combined is 250 mg/day (45). However, intake recommendations established by national authorities vary between different European countries ranging from 200 mg to 500 mg per day for 20:5n-3 plus 22:6n-3 (45). In the UK, the recommended daily intake of 20:5n-3 and 22:6n-3 endorsed by the Scientific Advisory Committee on Nutrition (SACN) is 450 mg (46). The evidence supporting recommendations for 20:5n-3 and 22:6n-3 intake provided by different international and national bodies is mostly related to lower risk of cardiovascular disease (CVD). However, clinical benefits are not consistent in all studies and the relationship between n-3 PUFAs and risk of CVD may vary in populations with differing prevalence of CVD, which may explain why recommendations differ between countries. Furthermore, recommendations may be influenced by dietary habits. For example, in countries with high habitual fish intake, considerations for recommended 20:5n-3 and 22:6n-3 consumption may not carry such importance.

1.5.2 Overview of the health Benefits relating to n-3 PUFA consumption

n-3 PUFAs have many important effects on cell and tissue function and therefore it is not surprising that they have been linked to many health benefits. n-3 PUFAs are critical for optimal structure and function of biological membranes. The fatty acid composition of the membrane has a strong influence on its fluidity and therefore protein movement, which can ultimately affect cell function (48, 49). A high level of 22:6n-3 is found in the membrane phospholipids of the brain and retina where is important in signalling pathways and neurological function (50, 51). Furthermore, n-3 PUFAs have important effects on the production of inflammatory mediators, such as eicosanoids (52) and the regulation of gene expression (53), consequently influencing cell function. These mechanisms of action of n-3 PUFAs are discussed in more detail in section 1.10.
The first evidence for the health benefits associated with n-3 PUFA consumption came from studies on Inuit populations in Greenland, Northern Canada and Alaska where it was documented that they had much lower cardiovascular mortality (54, 55). It was suggested that the high levels of longer chain n-3 PUFA consumed in their diet, as a result of the regular intake of seal meat and whale blubber, was having a protective action (56). Evidence reviewed from epidemiological studies and intervention trials suggests that long chain n-3 PUFA consumption may lower the risk of cardiovascular disease (CVD) (57). Supplementation with 20:5n-3 and 22:6n-3 was reported to reduce the rate of death from cardiac causes by 20% in a meta-analysis of randomised control trials involving patients with cardiac disease (58). Another meta-analysis of cohort studies and clinical trials reported that a combined intake of 250 mg of 20:5n-3 and 22:6n-3 per day lowered the risk of fatal coronary heart disease by 36%, with no further benefit from higher intakes (59). In contrast, some more recent studies have not reported beneficial effects of n-3 PUFA supplementation on CVD outcomes (60). For example, neither supplementation (for 40 months) with 18:3n-3 (2 g/day) or 20:5n-3 and 22:6n-3 (400 mg/day) reduced the rate of major cardiovascular events (fatal and non-fatal CVD and particular cardiac intervention), in a study of 4837 patients who had suffered a myocardial infarction (61). Another study of 12,513 patients with multiple CVD risk factors but not myocardial infarction found no effect of daily supplementation with 1 g/day n-3 PUFA (providing at least 850 mg of 20:5n-3 and 22:6n-3) on cardiovascular mortality and morbidity over a 5 year period (62). A systematic review of the association between n-3 PUFA and CVD risks factors or outcomes was published in 2016. The review included 61 randomized control trials and 37 longitudinal observational studies. The authors concluded that there was high strength of evidence that high marine oil intake does not affect risk of major adverse cardiovascular events, all-cause death, sudden cardiac death, coronary revascularisation or blood pressure (63). Furthermore, a meta-analysis published in 2012, including 14 randomised, double-blind, placebo controlled trials involving 20,485 patients with a history of CVD, concluded that there was insufficient evidence that supplementation with n-3 PUFA (20:5n-3 and 22:6n-3) reduced the risk of overall cardiovascular events (64). In summary, although earlier studies reported preventative effects of n-3 PUFA on CVD outcomes, the evidence remains inconclusive. Possible reasons for conflicting findings between earlier and more recent studies might include differences in supplementation periods and doses, sample sizes, dietary factors and improvements in drug treatments.

In addition to effects on CVD, health benefits relating to n-3 PUFA consumption have been reported for a number of different conditions. The effect of n-3 PUFA on the function of cell types involved in inflammation and the production of inflammatory mediators (see section 1.10) suggest they act in an anti-inflammatory and inflammation resolving manner (65). Accordingly, n-3 PUFA have been reported to exert beneficial effects in inflammatory conditions such as rheumatoid arthritis,
inflammatory bowel disease and asthma (66-69). Furthermore, there is evidence that n-3 PUFAs are important for neurological development in infants and protecting against cognitive decline with ageing (57). Benefits have also been shown in mental health and behavioural conditions such as depression, attention deficit hyperactivity disorder (ADHD) and schizophrenia (57).

There is less evidence for the protective effects of 18:3n-3. Nevertheless, the risk of fatal coronary heart disease was found to be 21% lower in subjects consuming high amounts of 18:3n-3 compared with low intakes in a meta-analysis of five prospective cohort studies (70). In such studies, it is difficult to isolate the activity of 18:3n-3 itself from the products formed by its conversion. However, the impact that 18:3n-3 deficiency has on neurological function strongly suggests that it has an important role as a precursor for the synthesis of longer chain n-3 PUFA, particularly 22:6n-3. Evidence has been obtained from studies on visual function in rhesus monkeys which have a similar retinal anatomy and visual capabilities to humans (71). In a study on infant rhesus monkeys fed an 18:3n-3 deficient diet, 22:6n-3 in plasma phospholipids was progressively depleted and visual function was also shown to decline; visual acuity was half that of control infants at weeks 8 and 12 of the diet (72). Low levels of 22:6n-3 were also found in the tissues of infant rhesus monkeys fed an 18:3n-3 deficient diet from birth and whose mothers had also been fed an 18:3n-3 deficient diet during pregnancy (73). The deficient animals showed impaired visual function for their age (73). Furthermore, in a human case study, a six year old child receiving parenteral nutrition that was deficient in 18:3n-3 experienced peripheral neuropathy and blurred vision. These neurological symptoms disappeared when the diet was enriched with 18:3n-3 (74). A study in rats showed that animals raised on n-3 deficient diets had 80% less 22:6n-3 in retinal rod outer segment (ROS) membranes and this correlated with reduced G-protein-coupled receptor (GPCR) signalling efficiency (75).

1.5.3 Measuring n-3 PUFA status and synthesis

Accurate assessment of n-3 PUFA status and endogenous synthesis is important for determining how these factors relate to health and disease. When measuring fatty acid synthesis it is important to consider the type of tissue and lipid fraction the fatty is being measured in and the measurement technique that is being used. Measurement of fatty acids status and synthesis in humans often requires biological samples that are easy to access and non-invasive, such as blood cells and plasma. The most commonly reported tissues for measuring fatty acids in the literature include subcutaneous adipose tissue from the buttock or abdomen, plasma, platelets and erythrocytes (76).

The measurement of a fatty acid in a particular tissue reflects both the intake and the processes the fatty acids have been through in the body; for example, absorption, transport, uptake into
target tissues, metabolism from storage sites or excretion and also endogenous synthesis (77). Exchanges of fatty acids within and between lipid classes in tissues and blood ensures the widespread distribution of fatty acids throughout the body (76). Consequently, measurement of a fatty acid within a particular tissue represents a complex mixture of processes and will include fatty acids that have originated from the diet and from endogenous synthesis.

1.5.3.1 Measurement of n-3 PUFA synthesis using stable isotope tracers

Stable isotope tracer studies provide a powerful approach for measuring n-3 PUFA metabolism in humans, animal models and in vitro systems. The use of 18:3n-3 labelled with stable isotopes such as carbon 13 \[^{13}\text{C}\] or deuterium \[^{2}\text{H}\] has enabled detailed investigations into 18:3n-3 metabolism including its conversion to longer chain n-3 PUFA (by measuring labelled elongation products) and the extent of β-oxidation (by measuring labelled CO\(_2\) on breath or \(^{2}\text{H}\) in plasma water). The use of stable isotopes also overcomes the health hazards associated with using radioisotopes that were traditionally used to measure fatty acid metabolism.

The introduction of gas chromatography (GC) in the 1950s and its subsequent development gave rise to the first convenient means of quantifying stable isotope tracers in complex biological mixtures. This approach relied on the development of sophisticated mass spectrometry (MS) technology techniques. More recently, GC coupled to a combustion furnace followed by isotope ratio mass spectrometry (GC-C-IRMS) has become a popular technique for high precision compound specific isotope analysis of fatty acids. This technique permits measurement of isotope ratios at precisions sufficient to detect differences due to natural processes in all compounds eluted in sufficient quantities from the GC (78). In this process GC separates the analyte from a mixture of compounds, the analyte is then oxidatively combusted and the analyte gases are subsequently ionised and detected allowing the stable isotope ratio to be measured relative to a standard of known isotopic enrichment.

A disadvantage of IRMS is the fatty acid structure cannot be determined as this technique yields no molecular ion data. Furthermore, fatty acids labelled with stable isotopes can be expensive and coupled with the time and effort involved with using stable isotope tracer techniques they become less attractive for use in large-scale studies. Consequently many studies have small numbers of subjects, which raises the question of reliability particularly as individuals often have large natural variability (78). Furthermore, the \[^{13}\text{C}\] content of different foods can vary which could lead to natural variations between individuals. In order to minimise the impact of this on results, the baseline \[^{13}\text{C}\] enrichment must be subtracted. The average natural abundance of \[^{13}\text{C}\] in food is about 1.1% but this varies depending on the food source (78).
1.5.3.2 Measuring n-3 PUFA synthesis using the product to precursor ratio

Another technique that has been used to estimate n-3 fatty acid synthesis and therefore desaturase activity is the ratio of product, for example, 20:5n-3 or 22:6n-3 to precursor (18:3n-3). This ratio is also referred to as the desaturation index. The product to precursor ratio has been used in several studies (79-82) and is well established as a surrogate measure of desaturase activity. It is a particularly popular measurement in studies where it is used as a predictor of metabolic and CVD and mortality (83).

The main advantage of using this technique to measure fatty acid synthesis is that fatty acid profiles and concentrations are relatively simple and cost-effective measurements compared with stable isotope tracer techniques. However, a major issue with this is that the fatty acid composition of the cell membrane is influenced by both dietary intake and metabolic pathways and therefore can only be a crude measure of synthesis. High dietary content of the fatty acid being measured can dilute the pool of endogenously synthesised product therefore causing inaccurate measurement. Furthermore, there is no evidence that this technique has been validated against direct measurements of synthesis using stable isotopes. Su and Brenna (1998), reported a non-tracer method for measuring desaturase activity using GC with flame ionisation detection (GC-FID) (84). They simultaneously measured delta-6 and delta-9 desaturase activities in microsome preparations using a tracer and non-tracer method and found them to be consistent. However, their measurements were based on the rate of decrease in precursor (or increase in product) over time and were not merely a measure of the product to precursor ratio. A recent study reported a poor relationship between product to precursor ratios and delta-6 desaturase activity and concluded that coupled desaturation-elongation products more accurately reflected activity (22).

1.6 Evidence for 18:3n-3 conversion in humans

Findings from both stable isotope studies and studies where the amount of 18:3n-3 in the diet was increased have given complementary results about the metabolic fate of 18:3n-3. There has been consistent evidence from studies that increasing 18:3n-3 intake results in an increase in the proportion of 20:5n-3 in plasma and cell lipids. Burdge, 2006, plotted 18:3n-3 intake in g/day against the % change in 20:5n-3 and 22:6n-3 in plasma phospholipids using data from a number of different studies and found a significant positive correlation with 20:5n-3 (85). Increasing 18:3n-3 consumption has also been shown to significantly increase the proportion of 22:5n-3 in plasma and cell lipids (86-90) but such studies have shown no significant increase in the proportion of 22:6n-3; in some studies 22:6n-3 has been shown to decrease significantly (89, 91). However, most of these
studies were done in men or mixtures of men and women and therefore do not give a clear indication of the ability of women to synthesise 22:6n-3.

Evidence for the conversion of 18:3n-3 in humans has also come from stable isotope studies where participants consume 18:3n-3 labelled with stable isotope. Overall, data from these studies suggests that the conversion of 18:3n-3 to 20:5n-3 and 22:5n-3 is low, with estimates ranging from 0.2% to 6% and 0.13% to 6%, respectively (85). The conversion of 18:3n-3 to 22:6n-3 has been shown to range from undetectable (92) up to 4% (93), although 4% appears to be an exception. The general consensus from stable isotope tracer studies is that conversion is actually 0.05% or less (85).

1.7 Regulation of 18:3n-3 conversion

1.7.1 Feedback inhibition and competition

Stable isotope studies have shown that increasing consumption of 20:5n-3 and 22:6n-3, individually or combined, results in feedback inhibition of the pathway. In one study, supplementing the diet with 22:6n-3 (6.5 g/day) resulted in a 76% reduction in 20:5n-3 synthesis and an 86% reduction in 22:6n-3 synthesis (94). Increased consumption of 20:5n-3 and 22:6n-3 has also been shown to decrease the synthesis of 22:6n-3 in women (95, 96) and of 20:5n-3 and 22:5n-3 in men (97). Another stable isotope study showed that high consumption of 18:3n-3 significantly reduced conversion to 20:5n-3; 22:5n-3 and 22:6n-3 synthesis also tended to be lower (98). This data suggests that increasing the consumption of n-3 PUFA down regulates their synthesis from 18:3n-3 although how this happens is not fully understood. A suggested mechanism involves transcriptional regulation of FADS2 (6). It is also possible that high levels of 18:3n-3 intake may reduce 22:6n-3 synthesis due to the role of delta-6 desaturase in two stages of the pathway, thereby increasing substrate competition.

1.7.2 Gender differences in n-3 PUFA synthesis

Evidence suggests that there are gender differences in 18:3n-3 metabolism. Two comparable stable isotope tracer studies, one in women approximately 28 years of age (99) and one in men approximately 36 years of age (100), found that 20:5n-3 synthesis was 2.5 times higher and 22:6n-3 synthesis greater than 200 times higher in women compared with men. Further evidence comes from kinetic analysis by Pawlosky et al., (2003) who showed that conversion of 22:5n-3 to 22:6n-3 had a rate constant coefficient that was approximately 4-fold higher in women compared with men who were both consuming a beef based diet (95, 96). Furthermore, the amount of 22:5n-3 used for 22:6n-3 synthesis was nearly 3-fold greater in women compared with men. However, differences
between men and women were not seen when they were both consuming a fish based diet. A major limitation of both these studies were the small number of participants (six or less in each group) and the studies by Burdge and Wootton, 2002 were conducted at different times (99, 100).

Findings from several studies suggest that the differences between men and women regarding the conversion 18:3n-3 could be explained by the effect of female hormones. It has been reported that 22:6n-3 synthesis in young women using the oral contraceptive pill containing 17α-ethynylestradiol (EE₂) was almost 3-fold higher than in women that did not use it (99). Another study found that oestrogen based hormone replacement therapy in post-menopausal women resulted in greater plasma dihomo-γ-linolenic (20:3n-6) and arachidonic acid (20:4n-6) concentrations (101). This supports the suggestion that oestrogen may increase the activity of the n-3 and n-6 pathway although only n-6 fatty PUFA were found to be increased. In a study by Giltay et al., (2004), the concentration of 22:6n-3 in the plasma CE fraction was reported to be significantly higher in women than in men who were both consuming diets with controlled 18:3n-3 and energy content (102). In this study 22:6n-3 status was also found to be higher in women taking oral contraceptives (0.58%) than those who were not (0.53%) but this was not found to be significant (P = 0.08). Furthermore, 22:6n-3 in plasma CE in male to female transsexuals receiving EE₂ was significantly increased (by 42%) whereas in female to male transsexuals receiving testosterone 22:6n-3 was significantly decreased (by 20%). In another study, administration of raloxifene (an estrogen receptor modulator) or hormone replacement therapy (HRT) increased the proportion of 22:6n-3 in plasma CE by a mean of 22.1% and 14.9%, respectively, compared with the placebo group (103). These studies had the advantage of having a large number of participants; however, 22:6n-3 is a minor component of the CE pool and is much more abundant in phospholipids.

The above studies suggested that oestrogen could be responsible for increasing 22:6n-3 synthesis. However, in a study investigating n-3 PUFA synthesis in the human hepatocarcinoma cell line (HepG2), progesterone, not EE₂, significantly increased conversion of deuterated 18:3n-3 to 20:5n-3, 22:5n-3 and 22:6n-3 and increased the expression of FADS2, FADS1, ELOVL2 and ELOVL5. A limitation of this study is that these measurements were made in a cell line, which may be metabolically different to primary cells. However, progesterone also increased the expression of these genes in primary human hepatocytes and this was statistically significant for FADS2 (104). In another study, this time in rats, oestrogen and progesterone showed a significant positive correlation with hepatic Fads2 mRNA expression (105). Furthermore, the HRT administered in the study by Giltay et al., (2004) contained conjugated equine oestrogens (CEE) and also a synthetic form of progesterone – medroxyprogesterone acetate (MPA) (103). Collectively, the findings from these studies indicate gender differences in n-3 PUFA status and synthesis might involve the action
of sex hormones. However, it is less clear how the relative roles of oestrogen and progesterone influence the pathway and the mechanisms behind this.

It has been suggested that greater 18:3n-3 conversion in women may be serving a biological role in meeting the demands of the developing foetus for 22:6n-3. In humans, the concentration of 22:6n-3 in plasma PC has been shown to significantly increase between 16 weeks and 40 weeks gestation (106). In rats, 22:6n-3 has been shown to increase in plasma and also the liver during pregnancy (105). Rapid synthesis of brain tissue occurs during the last trimester of pregnancy and consequently the demand for 22:6n-3 is very high (107). Consequently, it has been suggested that the supply of 22:6n-3 to the developing foetus by the mother may be of particular importance (108).

1.7.3 Gender differences in 18:3n-3 \( \beta \)-oxidation

There is some evidence that the reported gender differences in 22:6n-3 status and 18:3n-3 conversion could in part, be explained by \( \beta \)-oxidation; the process by which fatty acids are broken down for energy production, primarily in the mitochondria. In this process, fatty acids are activated by conversion to a fatty acyl CoA and cleavage of the chain generates acetyl CoA, which is used to yield energy by the tricarboxylic acid (TCA) cycle or is used in fatty acid synthesis de novo.

Partitioning of \([^{13}C]18:3n-3\) towards \( \beta \)-oxidation has been shown to be lower in young women compared with young men at 22% (99) and 33% (100) of the administered dose, respectively, over the same time period (24 h). Furthermore, in a study in older men, mean proportion of the administered dose of \([^{13}C]18:3n-3\) recovered on breath as \(^{13}CO_2\) over 24 h was 33.8% (97). The higher level reported in men may be due to the differences between men and women in the mass of tissues such as skeletal and cardiac muscle, liver and kidney, where \( \beta \)-oxidation pathways are highly active (85). It could also be due to differences between men and women in the use of fat as an energy, which has been reported to be greater in men (109, 110). However, a major limitation of the studies reporting gender differences in 18:3n-3 \( \beta \)-oxidation is that there was no correction for loss of stable isotope label in the TCA cycle (111) and therefore the level of \( \beta \)-oxidation was probably underestimated.

18:3n-3 has been shown to be the preferred substrate for \( \beta \)-oxidation compared with other fatty acids. One study in males demonstrated that, following ingestion of \([^{13}C]\) labelled fatty acids (16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3), recovery of \(^{13}CO_2\) on the breath over a period of 9 h was greater for 18:3n-3 than the other fatty acids (112). Preference for 18:3n-3 as a substrate for \( \beta \)-oxidation has also been demonstrated in rats (113) and more specifically, 18:3n-3 has been shown to be the preferred substrate for the rate limiting enzyme in mitochondrial fatty acid \( \beta \)-oxidation (114).
The carbon released by β-oxidation can also be recycled in de novo fatty acid synthesis. In animals, labelled 18:3n-3 has been shown to be recycled into brain SFA and MUFA (115). In humans, carbon recycling of labelled 18:3n-3 into SFA and MUFA measured in plasma lipids was reported to be 20% greater in men compared with women (116). Furthermore, the recycling of carbon from 18:3n-3 into SFA and MUFA was greater than the conversion to longer chain n-3 PUFA in men, whereas in women it was the opposite. In summary, gender differences may be due in part, to differential partitioning towards β-oxidation in men and women therefore affecting the availability of 18:3n-3 for conversion to longer chain n-3 PUFA. This may be made more pronounced by the fact that 18:3n-3 is a preferred substrate for β-oxidation.

1.7.4 n-3 PUFA synthesis and ageing

Many of the health benefits relating to n-3 PUFA could be important for healthy ageing. The conversion of 18:3n-3 to longer chain n-3 PUFA and the extent of partitioning towards β-oxidation has been studied in older men (mean age 52 years) using the stable isotope tracer [U-13C]18:3n-3 (97). The proportion of labelled 18:3n-3 recovered as 13CO2 in breath was calculated as being comparable to studies involving younger men; however, the proportion of 18:3n-3 converted to 20:5n-3 and 22:5n-3 was between three and six times lower in the older men (97, 100, 117). The amount of 22:6n-3 synthesised did not differ between younger and older men but his may have been due to the very low synthesis of 22:6n-3 in men making any differences difficult to detect. Another study in humans found a decline in delta-6 desaturase activity with age and this was greater in women than in men (118). However, one study found no evidence that desaturase function decreased in ageing (post-menopausal) females (119). It should be noted that the last two studies mentioned measured the ratio of product to precursor as a way of estimating enzyme activity and not direct measures of synthesis using stable isotope tracers. Further evidence for how the activity of the n-3 pathway changes with age comes from studies in male rats that reported delta-6 desaturase activity in liver microsomes decreased with age (120, 121). Overall, less is known compared with gender differences about how ageing affects the conversion of 18:3n-3 to longer chain n-3 PUFA. Studies in humans and rats suggest it could decrease with age but more evidence is needed.

1.7.5 Single nucleotide polymorphisms

A number of studies have reported associations between single nucleotide polymorphisms (SNPs) and n-3 and n-6 PUFA levels. SNPs, a common type of genetic variation, are defined as a difference in a single nucleotide at a specific position in the genome. A study of 18 SNPs in 727 Caucasian adults reported strong associations between variants in the FADS2 and FADS1 genes and n-6 and
n-3 PUFA levels in serum phospholipids (122). Subjects carrying the minor alleles of 11 SNPs exhibited higher levels of 18:2n-6 and 18:3n-3 and decreased levels of their respective conversion products, 18:3n-6 and 20:4n-6 and 20:5n-3 and 22:5n-3 suggesting SNPs were altering desaturase activities. Interestingly, these SNPs were also associated with elevated levels of 20:2n-6, a product in the alternative delta-8 desaturation pathway, perhaps providing further evidence that delta-6 desaturase catalyses delta-8 desaturation (see section 1.4.4). Another study, this time involving analysis of 13 SNPs in 658 adults with CVD, reported similar findings (123). Minor alleles of individual SNPs were associated with higher levels of 18:2n-6, 20:2n-6 and 18:3n-3 and lower levels of 20:4n-6 in serum phospholipids and erythrocyte membranes; the strongest association reported was with 20:4n-6. No significant association was found for the n-3 PUFAs 18:4n-3, 20:5n-3 or 22:6n-3, which may have been due to no association with causative genetic factors or due to other factors such as diet (123). A study of 1,144 European adolescents, reported associations between SNPs in the FADS1 and FADS2 genes and delta-6 and delta-5 desaturase activities (124). The minor alleles of nine SNPs were associated with lower delta-5 desaturase activity, whereas the minor allele of one SNP was associated with higher delta-6 desaturase activity. However, ratios of n-6 PUFAs were used as proxy measures of desaturase activity in this study, which has questionable accuracy (see section 1.5.3.2).

Since many of the SNPs analysed in the FADS1-FADS2 gene cluster are located in untranslated regions (UTRs), introns and intergenic regions, it has been suggested that a potential function of these SNPs is the regulation of transcription. This is supported by a functional study that showed variation in a specific SNP (rs968567) influenced promoter activity and binding of the transcription factor ELK1 (125). Promoter activity increased with the minor T allele (125) and in another study this minor allele of FADS2 was shown to be associated with higher levels of delta-6 desaturase activity (124).

1.8 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) are defined as any peripheral blood cell having a round nucleus. These cells are a critical component of the immune system, collectively having important roles in innate (responses not specific to a particular pathogen) and adaptive (refers to the response to a specific antigen) immune responses (126, 127). PBMCs consist of lymphocytes (T-lymphocytes, B-lymphocytes and Natural Killer (NK) lymphocytes), monocytes and dendritic cells. In humans, the frequencies of these populations vary across individuals but PBMCs are typically composed of 70-90% lymphocytes and 10-20% monocytes, while dendritic cells are rare, accounting for only 1-2%. The lymphocyte population consists of approximately 70-85% T-lymphocytes, 5-10% B-lymphocytes and 5-20% NK cells (128). B-lymphocytes mature in the bone marrow and are the cells responsible
for producing antibodies, also called immunoglobulins (Ig). The antibodies produced by B-lymphocytes recognise individual antigens and function to neutralise and stimulate removal of pathogens. This type of immune protection is termed the humoral response, a component of the adaptive immune system (129). Monocytes also mature in the bone marrow and are the largest type of leukocyte. Monocytes can differentiate into macrophages, large phagocytic cells that play a critical role in innate immunity (126). Natural killer (NK) cells are large granular lymphocyte-like cells with important functions in innate immunity. They lack antigen-specific receptors but are capable of detecting and killing tumour and virus-infected cells. T-lymphocytes mature in the thymus and display a central role in cell-mediated immunity, a component of the adaptive immune system directed specifically at intracellular pathogens (127). T-lymphocytes recognise and respond to specific foreign antigens and can be further categorised into distinct sub-sets based on their effector function. The most defined of these sub-sets are helper T-lymphocytes (Th-lymphocytes) and cytotoxic T-lymphocytes (CTLs), characterised by the CD4 or CD8 molecules, respectively, on the cell surface. The activation of T-lymphocytes is discussed in more detail below.

1.9 T-lymphocyte activation

Resting (naïve) T-lymphocytes must be activated to proliferate and differentiate into effector cells before they can take part in an immune response. This response also generates memory cells that facilitate an accelerated response to subsequent challenge by the same antigen. The primary signal for T-lymphocyte activation involves recognition of specific antigens by a surface expressed, highly variable T cell receptor (TCR). The TCR cannot bind free antigen directly and instead recognises a foreign peptide bound to a major histocompatibility complex (MHC) protein on the surface of an antigen presenting cell (APC). There are two types of MHC: MHCI and MHCII. MHCI is expressed on virtually all somatic cells and presents endogenously generated peptides to CTLs (130). MHC II is found on macrophages, B-lymphocytes and dendritic cells and presents processed antigens derived primarily from exogenous sources to Th-lymphocytes (131). The TCR lacks the ability to signal independently and requires the CD3 co-receptor to transduce signals from the TCR into the lymphocyte. The presence of CD3 is a defining feature of T-lymphocytes and therefore is an effective marker used for their identification (132). The CD4 and CD8 co-receptors also play important roles in the signalling process acting to stabilise the interaction between the TCR and antigenic peptide-MHC complex (Figure 1.3). In addition, activation also requires a second signal involving binding of the co-stimulatory receptor CD28 on the T-lymphocyte with B7 molecules on the surface of the APC (133). The signals originating from the TCR trigger an intracellular signal transduction cascade, transmitting signals away from the membrane into the cytoplasm and nucleus resulting in initiation of distinct transcriptional programmes that allow T-lymphocytes to
execute their effector functions. One well defined model for signal transduction involves activation of phospholipase C (PLC) resulting in hydrolysis of membrane phospholipid phosphatidylinositol 4,5 bisphosphate. This generates the secondary messengers inositol trisphosphate (IP$_3$) and diacylglycerol (DAG), which increase intracellular calcium and activate protein kinase C (PKC), respectively (134).

In response to stimulatory signals, T-lymphocytes secrete cytokines that function in promoting T-lymphocyte proliferation and differentiation. For example, stimulated T-lymphocytes simultaneously synthesise interleukin-2 (IL-2) receptors and secrete IL-2. The binding of IL-2 to its receptors activates transcriptional pathways that promote T-lymphocytes to proliferate and differentiate into effector cells. Cytokines secreted by activated T-lymphocytes also activate other cell types including B-lymphocytes, NK cells and macrophages (135).

![Figure 1.3. Schematic representation of the TCR engagement with peptide-antigen MHC complex.](image)

APC, antigen presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor. Adapted from Gascoigne, 2006 (136).

### 1.9.1 T-lymphocyte activation and fatty acids

T-lymphocyte activation is a highly complex process involving multiple events. De novo synthesis and turnover of membrane phospholipids are among the earliest events to occur following T-lymphocyte activation (137-139). These events result in alterations to the fatty acid composition of membrane phospholipids. Early studies on calf and rabbit lymphocytes demonstrated a rapid enrichment (occurring within 4 h after stimulation) in polyunsaturated acyl moieties of membrane phospholipids (137). Furthermore, experiments in cultured human T-lymphocytes demonstrated that levels of 18:1n-9 and the n-3 PUFA 22:5n-3 and 22:6n-3 increased following stimulation where
the n-6 PUFA 18:2n-6 and 20:4n-6 decreased (40). These changes mainly affected major phospholipids (PC and PE) as opposed to neutral lipids (40). This study also showed that membrane fluidity increased following activation; an event documented in a number of earlier studies (140-143). The authors suggested that the changes in fatty acid composition may contribute to the observed increase in membrane fluidity and could be important in the activation process (40).

Exogenous fatty acids are essential for in vitro activation and continued proliferation of T-lymphocytes (144, 145). In vitro experiments have demonstrated that activated T-lymphocytes increase the uptake of fatty acids, including the n-3 PUFAs 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3, when available, from the culture medium (40, 49). A recent study in CD4+ T-lymphocytes demonstrated that both fatty acid uptake and de novo fatty acid biosynthesis pathways are required for robust proliferation after antigenic stimulation; inhibition of either results in insufficient proliferation and inhibition of both leads to apoptosis (146). Furthermore, an early study demonstrated that activation of n-3 PUFA synthesis from 18:3n-3 occurs in T-lymphocytes stimulated to proliferate whereas in resting T-lymphocytes synthesis was negligible (147). The findings from this study are discussed in more detail in section 3.1. Importantly, no other studies since have investigated the activity or regulation of PUFA synthesis in activated T-lymphocytes. Interestingly, a significant level of n-3 and n-6 PUFA biosynthesis has been previously reported in a spontaneously dividing leukaemic T-lymphocyte cell line (148), which is reminiscent of the well-documented high level of de novo fatty acid synthesis observed in cancerous cells (149). De novo fatty acid synthesis occurs regardless of the level of exogenous fatty acids and serves important functions in membrane biosynthesis, energy storage and the production of signaling molecules in highly proliferating cancerous cells (149, 150). Less is known about the activity and importance of PUFA synthesis in cancerous cells, which is covered in more detail in section 6.1 in Chapter 6.

1.9.2 Stimulating lymphocyte proliferation in culture

Many studies have used cultured T-lymphocytes that have been stimulated to proliferate to investigate various T-lymphocyte responses. In vitro T-lymphocyte proliferation can be induced by various agents including monoclonal antibodies against certain surface antigens (e.g. CD3), chemicals such as calcium ionophore A23187 (increases cytosolic free Ca2+ concentration) and phorbol 12-myristate 13-acetate (PMA), which stimulates protein kinase C (135, 151), and mitogenic lectins such as concanavalin A (Con A) and phytohemagglutinin (PHA). Con A and PHA have been used extensively to study lymphocyte function in vitro. They exert their actions by binding and cross linking multiple cell surface glycoproteins, triggering intracellular events leading to mitosis (152).
Activated lymphocytes are known to express a number of molecules on their cell surface, which distinguish them from naïve T-lymphocytes. These molecules include receptor proteins, costimulatory molecules, adhesion molecules, chemokine receptors, and MHC class II molecules (153). Examples of receptor proteins known to be upregulated in proliferating lymphocytes include: CD69 (the early activation antigen), CD25 (the interleukin-2 receptor), CD71 (the transferrin receptor), and CD95 (the Fas receptor). Such activation markers are easily measured using fluorochrome labelled monoclonal antibodies and flow cytometric analysis and therefore the response to stimulation can be determined (153).

1.10 Effects of n-3 PUFAs on immune cell function

n-3 PUFAs have many important effects on cell and tissue function. Specifically, this section focuses on the effect of n-3 PUFAs on the functional activities of the cells of the immune system. Evidence indicates that n-3 PUFAs have vast effects on diverse immunological processes including cytokine production, lymphocyte proliferation, surface molecule expression, phagocytosis and apoptosis and NK cell activity and leukocyte chemotaxis (154, 155). Their mechanisms of action can involve alterations in the physical properties of the cell membrane such as fluidity and architecture, gene expression changes and changes to the production of lipid mediators (156). The effects of n-3 PUFAs on some of the processes mentioned above are described in more detail below.

1.10.1 Lymphocyte proliferation

A number of studies have investigated the effect of n-3 PUFAs on lymphocyte proliferation (summarised in Table 1.1). A study in rats demonstrated that feeding diets containing high levels (20%) of fish oil (rich in 20:5n-3 and 22:6n-3) results in suppressed proliferation of mitogen stimulated lymphocytes compared with diets rich in other fats (157). Mice fed diets (for 10 days) containing purified 20:5n-3 or 22:6n-3 (1%) showed suppressed lymphocyte proliferation in Con A stimulated cultures (158). Studies have also reported that n-3 PUFAs suppress the proliferation of human lymphocytes. Dietary supplementation with capsules containing 2.4 g of n-3 PUFA per day over a 3 month period reduced the proliferation of PHA stimulated PBMCs in women aged 51-68 years (159). However, this effect was not observed in young women in the same study. Furthermore, proliferation was only decreased in older women when lymphocytes were stimulated with PHA and not with Con A. Addition of 18 g per day of fish oil to the normal diet for 6 weeks suppressed PHA stimulated proliferation of PBMCs by 70% compared to pre-supplement level in men aged 21-39 years (160). However, proliferation was only significantly decreased when measured 10 weeks after cessation of supplementation and not at the end of the supplementation period at 6 weeks. Capsules containing a total of 1 g of 20:5n-3 and 22:6n-3 were shown to decrease
proliferation in Con A stimulated PBMCs from healthy older (aged 55-75 years) individuals (161). In another study, supplementing the diet of human volunteers with flaxseed oil (providing approximately 15 g of 18:3n-3 per day) has also been shown to suppress the proliferation of PHA and Con A stimulated PBMCs (162). In addition to dietary studies, experiments in which n-3 PUFAs were added directly to the culture media have also shown they exert an inhibitory effect on lymphocyte proliferation. In Con A stimulated rat lymphocytes, 18:3n-3, 20:5n-3 and 22:6n-3 supplementation of the culture medium decreased lymphocyte proliferation (163). The most potent inhibitor was shown to be 20:5n-3 and at concentrations above 50 µM the response was dose dependent (163). These findings were consistent with the results from a study in human PBMCs, where the proliferative response to Con A was inhibited following supplementation of the culture medium with 100 µM 18:3n-3, 20:5n-3 and 22:6n-3 (164).

Overall, the results from these studies suggest that n-3 PUFAs have an inhibitory effect on lymphocyte proliferation. However, many of these studies used doses of n-3 PUFAs far greater (see Table 1.1 for details) than the recommended daily intake for the UK of 450 mg (46). Furthermore, some more recent studies have reported either unchanged (87, 91) or increased (165, 166) in human lymphocyte proliferation following dietary n-3 PUFA supplementation, which conflicts the findings of the earlier studies. In conclusion, the effect of n-3 PUFAs on lymphocyte proliferation remains controversial. This could be due to several factors including differences in doses, supplementation periods or cell culture conditions. For example, in some studies the cell culture medium was supplemented with FBS, typically at 10% (v/v), whereas others supplemented with autologous plasma. FBS contains several lipids that have the potential to be hydrolysed and incorporated into the lymphocyte membrane; possibly disrupting the balance of fatty acids in the membrane induced by dietary supplementation or, in the case of cell culture supplementation studies, these fatty acids may compete with exogenous fatty acids under study. Furthermore, the composition of FBS varies considerably between different lots and, together with variation in the type and/or amount of serum added, this might account for some of the inconsistencies between studies.
### Table 1.1. Studies investigating the effects of n-3 PUFAs on lymphocyte proliferation (continued on pages 51-53)

<table>
<thead>
<tr>
<th>Subjects/Study design</th>
<th>Proliferation assay</th>
<th>Results</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>Animal study</td>
<td></td>
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<tr>
<td>Supplementation: dietary</td>
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<tr>
<td>Female mice (n = 7) fed diets enriched in 1% 20:5n-3 or 1% 22:6n-3 for 10 days (free access).</td>
<td>Splenic lymphocytes</td>
<td>Proliferation significantly suppressed by dietary 20:5n-3 (approximately 75%) and 22:6n-3 (approximately 80%) relative to safflower oil control (3%).</td>
<td>(158)</td>
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<tr>
<td></td>
<td>Mitogen: Con A (5 µg/ml)</td>
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<tr>
<td></td>
<td>Medium supplemented with 10% (v/v) FBS</td>
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<tr>
<td></td>
<td>Culture period: 96 h, final 6 h in presence of [³H] thymidine</td>
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<tr>
<td>Animal study</td>
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<tr>
<td>Supplementation: dietary</td>
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<tr>
<td>Male rats (n = 3-5) fed low fat (2.4% lipid) or high fat diets containing 20% of various test lipids (see results) for 10 weeks (free access).</td>
<td>Lymphocytes purified from spleen, thymus and mesenteric nodes</td>
<td>Spleen lymphocytes: 2.5% autologous plasma cultures - proliferation significantly lower in 20% fish oil group compared to low fat group. 10% FBS cultures – proliferation significantly lower in 20% fish oil group than in 20% hydrogenated coconut oil (HCO) group.</td>
<td>(157)</td>
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<tr>
<td></td>
<td>Mitogen: Con A (5 µg/ml)</td>
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<tr>
<td></td>
<td>Medium supplemented with 10% (v/v) FBS or 2.5% (v/v) autologous plasma</td>
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<td></td>
<td>Culture period: 48 h then further 18 h in presence of [³H] thymidine</td>
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<tr>
<td>Animal study</td>
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<tr>
<td>Supplementation: culture medium</td>
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<tr>
<td>Lymphocytes from male rats. BSA complexed 18:3n-3, 20:5n-3 and 22:6n-3 added to culture medium at various concentrations (33-495 µM). Experiments performed at least twice with 3-8 replications.</td>
<td>Cervical lymph node lymphocytes</td>
<td>All n-3 PUFAs inhibited proliferation at concentrations above 50 µM compared to albumin-alone controls. Above 50 µM the inhibitory effect was dose dependent. At most concentrations the order of inhibition was: 20:5n-3&gt;22:6n-3&gt;18:3n-3. Time-course experiments with 100 µM n-3 PUFAs showed that 20:5n-3 and 18:3n-3 were inhibitory after 42 h and 22:6n-3 after 54 h culture. In serum free conditions all n-3 PUFAs had an inhibitory effect compared with albumin-alone controls and was greater than observed in presence of serum.</td>
<td>(163)</td>
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<tr>
<td></td>
<td>Mitogen: Con A (5 µg/ml)</td>
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<td></td>
<td>Medium supplemented with 10% (v/v) FBS or no serum.</td>
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<td></td>
<td>Culture period: 48 h then further 18 h in presence of [³H] thymidine</td>
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<td>Human study</td>
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<tr>
<td>Supplementation: dietary</td>
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<tr>
<td>Healthy younger (aged 23-33 years) and older (age 51-68) women (n = 6 in each group). Diets supplemented with 2.4 g n-3 PUFA (1.68 g 20:5n-3 and 0.72 g 22:6n-3) per day for 3 months.</td>
<td>PBMCs</td>
<td>Proliferation significantly reduced by n-3 PUFA in older women (36% reduction after 3 months) but there was no significant change in younger women.</td>
<td>(159)</td>
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<tr>
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<td>Mitogen: PHA (5 µg/ml)</td>
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<td></td>
<td>Medium supplemented with 10% (v/v) HI autologous plasma</td>
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<td></td>
<td>Culture period: 72 h then further 4 h in presence of [³H] thymidine</td>
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Summary: 20:5n-3 and 22:6n-3 supplementation decreased proliferation at higher concentrations
<table>
<thead>
<tr>
<th>Subjects/Study design</th>
<th>Proliferation assay</th>
<th>Results</th>
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<tbody>
<tr>
<td><strong>Human study</strong></td>
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<td></td>
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<tr>
<td><strong>Supplementation: dietary</strong></td>
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</table>
| Healthy men, aged 21-39 years (n = 9). Diets supplemented with 18 g fish oil (2.75 g 20:5n-3 and 1.85 g 22:6n-3) per day for 6 weeks. | Cells: PBMCs  
Mitogen: PHA (0.5, 1, 2.4, 8 µg/ml)  
Medium supplemented with 5 % (v/v) HI autologous serum  
Culture period: 48 h then pulsed with \( [^3H] \) thymidine and cultured for further 24 h | 10 weeks after ending supplementation proliferation levels induced by 1 µg/ml and 8 µg/ml PHA were decreased by 78% and 70%, respectively, compared with pre-supplementation levels.  
**Summary:** fish oil supplementation decreased proliferation | (160) |
| **Human study**      |                     |         |     |
| **Supplementation: dietary** | | | |
| Healthy men and women aged 56-74 years. Diets supplemented daily with either 2 g 18:3n3, 720 mg 22:6n-3, 4 g fish oil (720 mg 20:5n-3 and 280 mg 22:6n-3) or placebo oil for 12 weeks. | Cells: PBMCs  
Mitogen: Con A (5, 15, 25, 50 µg/ml)  
Medium supplemented with 2.5% (v/v) autologous plasma  
Culture period: 48 h then further 18 h in presence of \( [^3H] \) thymidine | Fish oil treatment resulted in significant time-dependent decline in lymphocyte proliferation at all Con A concentrations. Mean decline after 12 weeks treatment was 55%, 65%, 59% and 66% at 5, 15, 25 and 50 µg/ml Con A, respectively. Change in proliferation after 12 weeks of fish oil treatment was significantly different to change in placebo group.  
Lymphocyte proliferation was not significantly affected by 18:3n-3 or 22:6n-3 treatments.  
**Summary:** fish oil supplementation but not 18:3n-3 or 22:6n-3 alone decreased proliferation | (161) |
| **Human study**      |                     |         |     |
| **Supplementation: dietary** | | | |
| Healthy men aged 21-37 years. 14 day stabilisation followed by 56 days on either basal diet (n = 5) or flaxseed diet (n = 5) then diets crossed over for further 56 days. | Cells: PBMCs  
Mitogen: Con A (5 and 25 µg/ml) and PHA (2 and 10 µg/ml)  
Medium supplemented with 10% (v/v) FBS  
Culture period: 66 h then further 6 h in presence of \( [^3H] \) thymidine | 18:3n-3 was 21-fold higher in flaxseed diet than basal diet. Lymphocyte proliferation significantly repressed by flaxseed diet in both Con A and PHA cultures.  
**Summary:** flaxseed supplementation decreased proliferation | (162) |
| **Human study**      |                     |         |     |
| **Supplementation: dietary** | | | |
| Healthy men, aged 25-45 (n = 10). Diets supplemented with 3 g fish oil (0.78 g 20:5n-3 and 1.85 g 22:6n-3) per day for 2 months. | Cells: PBMCs*  
Mitogen: Con A (5 µg/ml)  
Medium – no supplements detailed  
Culture period: 48 h then further 18 h in presence of \( [2^{-14}C] \) thymidine | Fish oil supplementation resulted in a 40% increase in lymphocyte proliferative capacity compared to pre-supplement levels. This response was reduced to normal levels after a 2 month wash out period.  
**Summary:** fish oil supplementation increased proliferation | (166) |
<table>
<thead>
<tr>
<th>Human study</th>
<th>Supplementation: dietary</th>
<th>Subjects/Study design</th>
<th>Proliferation assay</th>
<th>Results</th>
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<td>21 male a marathon runners aged 37 ± 2 years. Diets of 8 subjects supplemented daily with 3 g of fish oil containing 0.3 g 20:5n-3 or 1.5 g 22:6n-3 for 60 days.</td>
<td>Cells: PBMCs*&lt;br&gt;Mitogen: Con A (50 µg/ml)&lt;br&gt;Medium supplemented with 10% (v/v) FBS&lt;br&gt;Culture period: 48 h then further 18 h in presence of [2-14C] thymidine</td>
<td>Fish oil supplementation resulted in an increase in lymphocyte proliferation in marathon runners relative to control group both before (90%) and after a race (49%).&lt;br&gt;&lt;strong&gt;Summary: fish oil supplementation increased proliferation&lt;/strong&gt;</td>
<td>(165)</td>
</tr>
<tr>
<td>Human study</td>
<td>Supplementation: dietary</td>
<td>Healthy males (n = 8 per group) aged 18-39 years. Diets supplemented daily with 3.5 g 18:3n-3 or 0.44, 0.94 or 1.90 g of 20:5n-3 and 22:6n-3 for 12 weeks.</td>
<td>Cells: PBMCs&lt;br&gt;Mitogen: Con A (25 µg/ml)&lt;br&gt;Medium supplemented with 5% (v/v) autologous plasma&lt;br&gt;Culture period: 48 h then further 18 h in presence of [3H] thymidine</td>
<td>Lymphocyte proliferation did not differ among treatment groups at baseline or at the end of supplementation. Proliferation did decrease with time in all groups so that it was significantly lower at 12 weeks than baseline but was also significant for the placebo group.&lt;br&gt;&lt;strong&gt;Summary: supplementation with 18:3n-3 or 20:5n-3 and 22:6n-3 had no effect on proliferation&lt;/strong&gt;</td>
<td>(87)</td>
</tr>
<tr>
<td>Human study</td>
<td>Supplementation: dietary</td>
<td>150 healthy males and females aged 25-72. Randomly assigned to one of 5 interventions: placebo (n = 30), 4.5 g (n = 30) or 9.5 g (n = 31) 18:3n-3/day or 0.77 g (n= 30) or 1.7 g (n = 29) of 20:5n-3 and 22:6n-3 per day for 6 months.</td>
<td>Cells: PBMCs&lt;br&gt;Mitogen: Con A (5, 15, 25, 50 and 75 µg/ml)&lt;br&gt;Medium supplemented with 5% (v/v) autologous plasma&lt;br&gt;Culture period: 48 h then further 18 h in presence of [3H] thymidine</td>
<td>There was no significant effect of time or treatment on lymphocyte proliferation for any concentration of Con A used.&lt;br&gt;&lt;strong&gt;Summary: supplementation with 18:3n-3 or 20:5n-3 + 22:6n-3 had no effect on proliferation&lt;/strong&gt;</td>
<td>(91)</td>
</tr>
<tr>
<td>Human study</td>
<td>Supplementation: culture medium</td>
<td>Lymphocytes isolated from peripheral blood collected from healthy women (aged 22-51). BSA complexed 18:3n-3, 20:5n-3 and 22:6n-3 (100 µM) added to culture medium (n = 5 – 7).</td>
<td>Cells: lymphocytes&lt;br&gt;Mitogen: Con A (15 µg/ml) or PPD (10 µg/ml)&lt;br&gt;Medium supplemented with 10% (v/v) FBS&lt;br&gt;Culture period: 48 h then further 18 h in presence of [3H] thymidine</td>
<td>In Con A stimulated cultures 20:5n-3 caused the greatest inhibition of lymphocyte proliferation (85%), followed by 22:6n-3 (57%) and then 18:3n-3 (33%). The proliferative response to PPD was lower than for Con A. In PPD stimulated cultures only 20:5n-3 (75%) and 22:6n-3 (51%) caused a significant decrease in lymphocyte proliferation.&lt;br&gt;&lt;strong&gt;Summary: supplementation with 18:3n-3, 20:5n-3 or 22:6n-3 decreased proliferation&lt;/strong&gt;</td>
<td>(164)</td>
</tr>
</tbody>
</table>
### Table 1.1. (continued from pages 50-52)

<table>
<thead>
<tr>
<th>Subjects/Study design</th>
<th>Proliferation assay</th>
<th>Results</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td><strong>Human study</strong></td>
<td></td>
<td></td>
<td>(167)</td>
</tr>
<tr>
<td><strong>Supplementation: culture medium</strong></td>
<td>Cells: PBMCs*</td>
<td>Results were reported for 6 replicates from 4 different experiments. 20:5n-3 treatment at 25, 50, 75 and 100 µM or 22:6n-3 treatment at 50, 75 and 100 µM resulted in a dose dependent reduction in lymphocyte proliferation relative to control. 20:5n-3 and 22:6n-3 promoted cell death at concentrations of 50 µM and 100 µM or above, respectively. <strong>Summary: supplementation with 20:5n-3 or 22:6n-3 decreased proliferation</strong></td>
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<tr>
<td>Lymphocytes isolated from healthy blood obtained from blood bank. 20:5n-3 and 22:6n-3 in ethanol added to culture medium at increasing concentrations (12.5 - 400 µM).</td>
<td>Mitogen: Con A (5 µg/ml)</td>
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<td></td>
<td><strong>Medium</strong> supplemented with 10% (v/v) FBS</td>
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<tr>
<td></td>
<td><strong>Culture period:</strong> 48 h then further 18 h in presence of [2-14]C thymidine</td>
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</table>

*PBMCs* maintained in RPMI medium for up to 2 h to allow adherence of monocytes to plates and separation from lymphocytes. FBS, fetal bovine serum; Con A, concanavalin A; PHA, phytohemagglutinin; PPD, purified protein derivative of *Mycobacterium tuberculosis*; HI, heat inactivated.
Eicosanoids are a family of oxygenated derivatives of the 20-carbon PUFAs arachidonic acid (20:4n-6), dihomo-γ-linolenic acid (20:3n-6) and 20:5n-3. They are bioactive signalling lipids that have well established roles in the regulation of inflammation and immunity. The PUFA precursor for eicosanoid synthesis is released from the cell membrane usually by the action of phospholipase A2 (PLA2). Eicosanoids include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), lipoxins (LXs), hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs) and are produced by the action of cyclooxygenase (COX) or lipoxygenase (LOX) enzymes (Figure 1.4). The principle precursor for eicosanoid synthesis is 20:4n-6 due to its greater abundance in the membranes of most cells compared with 20:3n-6 and 20:5n-3. Immune cells are both important sources and regulatory targets of eicosanoids (154). An early study reported lymphocytes to be poor producers of eicosanoids (168) although more recent evidence indicates that T-lymphocytes express COX and LOX enzymes and are a source of eicosanoids (169). Eicosanoids can have both pro- and anti-inflammatory effects and therefore have a complex role in the regulation of immune and inflammatory responses. In general, eicosanoids derived from 20:4n-6 act in a pro-inflammatory manner, although this is an over-simplification since PGE$_2$, for example, has both pro- and anti-inflammatory effects, and LX$_A_4$ and LX$_B_4$ have anti-inflammatory effects (170, 171). The cells present, the nature of the activating signal, the timing of eicosanoid production and the sensitivity of target cells to the eicosanoid levels produced, all influence their overall effect (135).

Increased dietary intake of 20:5n-3 and 22:6n-3 from fish oil results in increased proportions of these fatty acids in immune cell phospholipids (172, 173). This has been shown to be accompanied by a decrease in membrane 20:4n-6 content resulting in lower substrate availability for conversion to eicosanoids (172). Furthermore, 20:5n-3 and 22:6n-3 competitively inhibit the metabolism of 20:4n-6 to eicosanoids and 20:5n-3 itself is able to act as a substrate for both COX and 5-LOX enzymes. Macrophages isolated from mice fed a fish oil diet were demonstrated to produce less PGE$_2$ and TXB$_2$ following stimulation compared to mice fed on non-fish oil based diets (174) and dietary fish oil supplementation has been shown to result in increased in vitro generation of 5-LOX products of 20:5n-3 (5-series LTs) in human neutrophils (175). The metabolism of 20:5n-3 gives rise to structurally different mediators from those produced by 20:4n-6 (e.g. PGE$_3$ instead of PGE$_2$ and LTB$_5$ instead of LTB$_4$), which are considered to be less biologically potent than their analogues (65, 154). One reason for the lower biological potency of 20:5n-3-derived eicosanoids is their receptors typically have higher affinity for 20:4n-6-derived mediators (176).
More recently, novel families of lipid mediators derived from 20:5n-3 and 22:6n-3 have been characterised. These include the E-series and D-series resolvins produced from 20:5n-3 and 22:6n-3, respectively, and protectins and maresins produced from 22:6n-3 (Figure 1.4). Synthesis involves the COX and LOX pathways. E-series resolvins are derived from 20:5n-3 by a multistep process involving cytochrome (Cyt) P450 and 5-LOX where D-series resolvins are generated from 22:6n-3 by 15-LOX and 5-LOX. Alternative pathways for E- and D-series resolvin synthesis involve aspirin-acetylated COX-2.

Resolvins, protectins and maresins have anti-inflammatory and inflammation resolving properties acting to control the magnitude and duration of inflammation (177). Consequently, these mediators are collectively termed specialised pro-resolving mediators (SPMs). Inflammation is part of a normal host response to infection and injury. Regulated inflammatory responses are essential to health but when uncontrolled they can lead to tissue damage and disease (65). n-3 PUFAs can exert important anti-inflammatory effects by a number of mechanisms in relation to lipid mediator synthesis: by decreasing membrane 20:4n-6 levels and capacity for 20:4n-6-derived eicosanoid synthesis, by increasing production of weakly inflammatory or anti-inflammatory eicosanoids derived from 20:5n-3 and by increasing the production of anti-inflammatory and inflammation resolving SPMs from 20:5n-3 and 22:6n-3 (170). As mentioned in section 1.5.2, n-3 PUFAs have been reported to have beneficial effects in certain inflammatory conditions including rheumatoid arthritis, inflammatory bowel disease and asthma (66-69).

![Figure 1.4](image-url)

**Figure 1.4. Overview of the synthesis of lipid mediators from 20:4n-6, 20:5n-3 and 22:6n-3.**
COX, cyclooxygenase; LOX, lipoxygenase; CytP450, cytochrome P450; PGs, prostaglandins; TXs, thromboxanes; LTs, leukotrienes; HETEs, hydroxyeicosatetraenoic acids. COX-2* indicates aspirin acetylated COX-2. Adapted from Buechler et al., 2017 (178) and Calder, 2010 (170).
1.10.3 Cytokine production

Some studies have reported that supplementing the diet of healthy humans with fish oil, providing ≥2 g per day of 20:5n-3 and 22:6n-3, results in decreased production of certain cytokines including TNF, IL-1 and IL-6 (179-181). Furthermore, a significant negative correlation between mononuclear cell 20:5n-3 content and TNF-α and IL-1β production in response to stimulation has been demonstrated (182). However, there have been discrepancies in findings between studies with some studies administering lower amounts (< 2 g per day) of 20:5n-3 and 22:6n-3 (87, 91), and also higher amounts (183, 184) reporting no effect on cytokine production. Feeding 13.7 g of 18:3n-3 per day for four weeks was shown to significantly decrease TNF-α and IL-1β production by 30% and 31%, respectively in stimulated human mononuclear cells (182). The same study showed that the effects of 18:3n-3 supplementation were more modest than the effects of 20:5n-3 and 22:6n-3; supplementation with 2.7 g per day of 20:5n-3 and 22:6n-3 decreased the production of TNF-α and IL-1β by 74% and 80%, respectively. In a different study, a diet high in 18:3n-3 (19.1 g per day for a 6 week period) was shown to inhibit the production of IL-6, IL-1β and TNF-α in PBMCs from human volunteers with moderate hypercholesterolemia (185). It is not known whether the inhibitory effects of 18:3n-3 on cytokine production in these studies was due to 18:3n-3 itself or its conversion to longer chain n-3 PUFAs. In a different study, supplementing the diet with a lower dose of 18:3n-3 (3.5 g per day over 12 weeks) did not alter the production of measured cytokines, including IL-6, IL-1β and TNF-α in stimulated mononuclear cells (87). If the effects of 18:3n-3 on cytokine production are mediated by its conversion to longer chain n-3 PUFA, then it might be that lower doses of 18:3n-3 result in insufficient levels of conversion products required for inhibitory activity. In summary, the relationship between n-3 PUFA supplementation and cytokine production is not entirely clear since different studies (summarised in Table 1.2) have reported conflicting results. As already discussed for the effect of n-3 PUFAs on lymphocyte proliferation, the discrepancies in findings might be due to differences in study design, including the amount of n-3 PUFAs administered and the time-period over which supplementation and analysis took place, or experimental conditions including the cell culture conditions and cytokine measurement techniques.
### Table 1.2: Studies investigating the effects of n-3 PUFAs on the production of cytokines (continued from pages 58-59)

<table>
<thead>
<tr>
<th>Subjects/Study design</th>
<th>Cytokine analysis</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human study</strong>&lt;br&gt; Supplementation: dietary&lt;br&gt; Healthy males (n = 9) aged 27-39 years. Diets supplemented with 4 g n-3 PUFA (containing 2.04 g 20:5n-3 and 1.40 g 22:6n-3) per day for 18 weeks.</td>
<td>PBMCs cultured for 4 h in presence or absence of LPS (10 µg/ml). No serum concentration given. IL-6 production measured in supernatants by ELISA.</td>
<td>n-3 PUFA treatment for 18 weeks significantly decreased basal (by 50%) and LPS-stimulated (by 46%) IL-6 production compared to pre-supplementation levels. Decreased IL-6 production observed after n-3 PUFA supplementation seemed to depend mainly on IL-6 produced by monocytes, which represented approximately 19% of PBMC population. <strong>Summary:</strong> 20:5n-3 and 22:6n-3 supplementation decreased production of IL-6.</td>
<td>(180)</td>
</tr>
<tr>
<td><strong>Human study</strong>&lt;br&gt; Supplementation: dietary&lt;br&gt; Healthy males received a daily antioxidant preparation (n = 8, mean age 30.3) or placebo (n = 8, mean age 30.6) for 12 weeks. Simultaneously, all subjects received a course of fish oil increasing in amount every 4 weeks, equivalent to a total 20:5n-3 and 22:6n-3 intake of 0.3, 1.0 and then 2.0 g/day.</td>
<td>PBMCs cultured for 24 h in presence or absence of LPS (15 µg/ml). Medium supplemented with 5% (v/v) autologous plasma. IL-6 and TNF-α production measured in supernatants by ELISA.</td>
<td>Fish oil supplementation resulted in significant reductions in the production of both TNF-α and IL-6 by unstimulated and stimulated PBMC in both fish oil only and fish oil plus antioxidant groups. There were no significant differences between fish oil plus antioxidant and fish oil only groups so groups were combined (n = 16) for further analysis. TNF-α and IL-6 production by unstimulated and stimulated PBMC conformed to a negative but 'U-shaped' dose response relationship with n-3 PUFA intake. Maximum inhibition was demonstrated at supplementary intake of 1 g/day. 20:5n-3 concentration in plasma and erythrocyte phospholipids were significantly negatively correlated with PBMC cytokine production. <strong>Summary:</strong> 20:5n-3 and 22:6n-3 supplementation decreased production of specific cytokines.</td>
<td>(181)</td>
</tr>
<tr>
<td><strong>Human study</strong>&lt;br&gt; Supplementation: dietary&lt;br&gt; Healthy males (n = 9) aged 21-39 years. Diets supplemented with 2.76 g 20:5n-3 and 1.86 g 22:6n-3 per day for 6 weeks.</td>
<td>PBMCs cultured for 24 h in presence of LPS (1 and 10 ng/ml) or PHA (3 µg/ml). Medium supplemented with 2% (v/v) autologous serum. IL-1α, IL-1β and TNF production in pooled cell lysates and supernatants determined by radio-immunoassays.</td>
<td>LPS (1 ng/ml): After 6 weeks supplementation there was a significant decrease in IL-1β (by 43%) production compared to baseline. Decreases were also observed for IL-1α (32%) and TNF (22%) but were non-significant. 10 weeks following the end of supplementation significant decreases were measured for IL-1β (61%), IL-1α (39%) and TNF (40%) compared to baseline. All returned to pre-supplementation levels after 20 weeks. Similar results were observed for cells treated with 10 ng/ml LPS. PHA (3 µg/ml): After 6 weeks supplementation there was a significant decrease in IL-1α (by 14%) production compared to baseline. 10 weeks following end of supplementation both IL-1α and IL-1β were significantly reduced, by 37% and 53%, respectively. <strong>Summary:</strong> 20:5n-3 and 22:6n-3 supplementation decreased production of specific cytokines.</td>
<td>(179)</td>
</tr>
<tr>
<td><strong>Human study</strong>&lt;br&gt; Supplementation: dietary&lt;br&gt; Healthy males (n = 8 per group) aged 18-39 years. Diets supplemented daily with 3.5 g 18:3n-3 or 0.44, 0.94 or 1.90 g of 20:5n-3 and 22:6n-3 for 12 weeks.</td>
<td>PBMCs cultured for 24 h in medium supplemented with Con A (25 µg/ml) or LPS (15 µg/ml) and 5% (v/v) autologous plasma. Supernatants were collected and cytokines measured by ELISA.</td>
<td>Production of TNF-α and IL-1β by PBMC stimulated with LPS and the production of IL-2, IL-4, IL-10 and IFN-γ by PBMC stimulated with Con A did not differ between treatment groups at baseline or at the end of supplementation and did not differ between time points within any treatment group. IL-6 production was significantly lower compared to baseline after 12 weeks supplementation with either 0.94 g/day (65% decrease) or 1.90 g/day (40% decrease) 20:5n-3 and 22:6n-3 and significantly lower than observed in the placebo group. <strong>Summary:</strong> Higher doses of 20:5n-3 and 22:6n-3 decreased IL-6 production.</td>
<td>(87)</td>
</tr>
</tbody>
</table>
Table 1.2. (continued from page 57)

<table>
<thead>
<tr>
<th>Subjects/Study design</th>
<th>Cytokine analysis</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human study</strong></td>
<td></td>
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<tr>
<td><strong>Supplementation:</strong></td>
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<tr>
<td><strong>Dietary</strong></td>
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<tr>
<td>150 healthy males and</td>
<td>PBMCs cultured for</td>
<td>Blood samples taken at 0, 3 and 6 months for analysis. PBMCs stimulated with LPS:</td>
<td>(91)</td>
</tr>
<tr>
<td>females aged 25-72.</td>
<td>24 h in medium</td>
<td>production of TNF-α, IL-1β, IL-6 and IL-10 did not differ among the groups at baseline or at the end of the intervention. PBMCs stimulated with Con A: production of IL-2, IL-4 and IFN-γ did not differ among the groups at baseline or at the end of the intervention. <strong>Summary: No effect of n-3 PUFA supplementation on cytokine production</strong></td>
<td></td>
</tr>
<tr>
<td>Randomly assigned to</td>
<td>supplemented with</td>
<td>Cytokine production was measured at baseline, 26 and 52 weeks after the start and at 4, 8 and 26 weeks after cessation of supplementation. Circulating concentrations of IL-1β, TNF-α and IL-1Ra were not significantly different between the 0 and 9 g/day fish oil groups at any time point. In all groups, as well as the placebo group, LPS stimulated secretion of IL-1β and IL-1Ra was higher during supplementation than afterwards. LPS stimulated secretion of IL-1β, TNF-α and IL-1Ra was not significantly different between the placebo group and treatment groups at any time point. <strong>Summary: No effect of n-3 PUFA supplementation on cytokine production</strong></td>
<td>(183)</td>
</tr>
<tr>
<td>one of 5 interventions:</td>
<td>Con A (25 µg/ml)</td>
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<tr>
<td>placebo (n = 30),</td>
<td>or LPS (15 µg/ml) and 5% (v/v) autologous plasma.</td>
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<td>4.5 g (n = 30) or</td>
<td>Supernatants were collected and cytokines measured by ELISA.</td>
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<td>9.5 g (n = 31)</td>
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<tr>
<td>18:3n-3/day or</td>
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<tr>
<td>0.77 g (n = 30)</td>
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<td>1.7 g (n = 29)</td>
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<tr>
<td>20:5n-3 or 22:6n-3</td>
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<tr>
<td>per day.</td>
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<tr>
<td><strong>Dietary</strong></td>
<td></td>
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<tr>
<td>58 healthy monkeys</td>
<td>Whole blood cytokine production measured in presence and absence of LPS (10 µg/ml). LPS added directly to blood and incubated for 24 h at 25°C. Cytokines measured in plasma by radioimmunoassay except IL-6, which was measured by ELISA.</td>
<td></td>
<td>(183)</td>
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<tr>
<td>aged 21-87 years.</td>
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<td>Diets supplemented</td>
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<td>with 0, 3, 6 or 9 g</td>
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<td>fish oil, providing</td>
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<tr>
<td>0, 1.06, 2.13 or 3.19</td>
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<tr>
<td>g/day n-3 PUFA for</td>
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<td>1 year.</td>
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<tr>
<td><strong>Dietary</strong></td>
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<tr>
<td>42 healthy volunteers</td>
<td>PBMCs cultured for</td>
<td>Cytokines measured immediately before or 4 weeks after supplementation. Cytokines measured for LPS stimulated cultures: TNF-α, IL-1β, IL-6, IL-8 and IL-10. Cytokines measured for Con A stimulated cultures: IL-2, IFN-γ, IL-10, IL-5, TNF-α and IL-4. There were no significant effects of time or treatment on the production of any of these cytokines. Summary: No effect of 20:5n-3 or 22:6n-3 supplementation on cytokine production</td>
<td>(184)</td>
</tr>
<tr>
<td>aged 23-65 years.</td>
<td>24 h in medium</td>
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<tr>
<td>Diets supplemented</td>
<td>supplemented with</td>
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<tr>
<td>with placebo, 4.7 g</td>
<td>5% (v/v)</td>
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<tr>
<td>g/day 20:5n-3 or 4.9</td>
<td>autologous plasma</td>
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<tr>
<td>g/day 22:6n-3 for</td>
<td>and either</td>
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<tr>
<td>4 weeks.</td>
<td>Con A (25 µg/ml)</td>
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<td></td>
<td>or LPS (15 µg/ml)</td>
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<tr>
<td></td>
<td>(15 µg/ml).</td>
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<tr>
<td></td>
<td>Cytokines analysed in supernatant by flow cytometry with cytometric bead arrays.</td>
<td></td>
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<tr>
<td><strong>Dietary</strong></td>
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<tr>
<td>20 male (aged 36-39)</td>
<td>Cytokines measured in serum and PBMC culture medium by ELISA. PBMCs cultured for 24 h in medium supplemented with TCH® serum replacement either with or without LPS (1 ng/ml and 10 ng/ml).</td>
<td>The high 18:3n-3 diet provided 19.1 g/day 18:3n-3 compared to 10.6 g/day and 2.3 g/day in the high 18:2n-6 and average American diets, respectively. Blood samples were collected and analysed at the end of each diet period. IL-6, IL-1β and TNF-α production by PBMCs were significantly lower with the 18:3n-3 diet compared to the 18:2n-6 or average American diets. PBMC production of TNF-α was inversely correlated with 18:3n-3 and with 20:5n-3 concentrations in PBMC lipids with the 18:3n-3 diet. <strong>Summary: 18:3n-3 supplementation decreased production of specific cytokines</strong></td>
<td>(185)</td>
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<td>and 3 female (aged</td>
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<tr>
<td>55-65), overweight or</td>
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<td>obese hypercholesterolemic volunteers assigned to a sequence of 3 experimental diets: high 18:3n-3, high 18:2n-6 and average American diet for 6 weeks.</td>
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</table>
**Table 1.2. (continued from pages 57-58)**

<table>
<thead>
<tr>
<th>Subjects/Study design</th>
<th>Cytokine analysis</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human study</td>
<td>PBMCs cultured for 24 h in medium supplemented with 5% (v/v) FBS and LPS (20 µg/ml). Cells were lysed, supernatants collected and cytokines measured by ELISA.</td>
<td>Average 18:3n-3 consumption was 13.7 g/day in the flaxseed group and 1.1 g/day in sunflower group. After 4 weeks of dietary change TNF-α and IL-1β production decreased by 30% and 31%, respectively in the flaxseed group but did not change in the sunflower group. After fish oil supplementation (i.e. at 8 weeks) cytokine synthesis had decreased in both groups: in flaxseed group, TNF-α decreased by 77% and IL-1β decreased by 81%, whereas in the sunflower group TNF-α decreased by 70% and IL-1β decreased by 78%. The flaxseed diet increased 20:5n-3 content in PBMC phospholipids, whereas the sunflower diet did not. There was a significant inverse relationship between cellular 20:5n-3 concentration and both TNF-α and IL-1β when results for both diets at 4 and 8 weeks were combined. <strong>Summary:</strong> 18:3n-3 supplementation and 20:5n-3 and 22:6n-3 supplementation decreased production of specific cytokines.</td>
<td>(182)</td>
</tr>
<tr>
<td>Supplementation: dietary</td>
<td>Healthy males aged 24-44 years. Diet supplemented with flaxseed oil high in 18:3n-3 (n = 15) or sunflower oil high in 18:2n-6 (n = 15) for 8 weeks. After first 4 weeks diets were supplemented with fish oil (1.62 g 20:5n-3 and 1.08 g 22:6n-3 per day).</td>
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</table>

FBS, fetal bovine serum; Con A, concanavalin A; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay
Regulation of gene expression may be one mechanism through which n-3 PUFA affect immune cell function. PUFAs can affect gene expression by regulating the activity or abundance of transcription factors. These transcription factors include peroxisome proliferator-activated receptors (PPARs), sterol regulatory binding proteins (SREBPs) and nuclear factor kappa B (NF-κB). PPARs are a family of ligand-activated nuclear receptors that play an important role in cell differentiation and various metabolic processes, particularly lipid and glucose homeostasis (186, 187). There are three PPAR isoforms: PPARα, PPARβ/δ and PPARγ. PPARs are activated by a broad range of ligands including eicosanoids and n-3 PUFAs, such as 18:3n-3, 20:5n-3 and 22:6n-3 (53, 188-190). Therefore, through activation of PPAR, n-3 PUFAs are able to affect specific aspects of metabolism and cell function. In addition to the well-studied effects of PPARs on the metabolic system, several pieces of evidence suggest that PPARγ and in some cases PPARα, are important regulators of cells of the immune system including T-lymphocytes, monocytes and macrophages, displaying modulatory roles in inflammatory responses (191-193). A recent study showed that activated CD4+ T-lymphocytes utilise exogenous fatty acids through MTORC1 (mechanistic target of rapamycin complex 1) mediated induction of PPARγ and its downstream fatty acid uptake target genes (146). Furthermore, the MTORC1-PPARγ pathway was demonstrated to be required for full activation and optimal proliferation (146).

n-3 PUFAs also affect SREBP dependent gene expression. SREBPs have important roles in regulating genes of fatty acid and cholesterol metabolism. Three SREBPs are recognised: SREBP1a, SREBP1c and SREBP2. SREBPs are regulated post transcriptionally: the inactive precursor form is located in the ER and is activated following translocation to the Golgi and release of the transcriptionally active amino-terminal fragment of SREBP (n-SREBP) (194). n-3 PUFAs can significantly decrease transcriptionally active concentrations of n-SREBP (195). It is not entirely clear how this happens but appears to involve a combination of mechanisms including decreased SREBP transport out of the ER and interactions with different steps of sphingolipid metabolism (194). SREBPs have been reported to serve important functions in activated T-lymphocytes. For example, SREBPs were found to be required for full activation of CD8+ T-lymphocytes, displaying specific importance in the rapid synthesis of membranes required for cellular growth (196). Furthermore, SREBP-1 induced fatty acid biosynthesis programmes were shown to be required for optimal proliferation in both murine and human CD4+ T-lymphocytes (146).

NF-κB is one of the key transcription factors involved in immune responses and plays an essential role in promoting T- and B-lymphocyte activation (197). In resting cells, NFκB is kept in an inactive form in the cytoplasm by inhibitor of NFκB (IκB) family members that prevent NFκB from moving
into the nucleus. Upon activation, IκB is phosphorylated by the IκB kinase (IKK) complex and subsequently degraded, which allows NFκB to move to the nucleus and activate target genes. Evidence indicates that n-3 PUFAs can downregulate the activity of NF-κB (198, 199). In human monocytic THP-1 cells stimulated with lipopolysaccharide (LPS), 20:5n-3 appeared to prevent NF-κB activation by preventing the phosphorylation of IκB-α (199).

1.10.5 Membrane properties

As mentioned in section 1.9.1, T-lymphocyte activation has been shown to be accompanied by an increase in membrane fluidity. An early study demonstrated that T-lymphocyte activation was associated with an increase in the 22:5n-3 and 22:6n-3 content of major phospholipids (40). The authors suggested that these fatty acid compositional changes might contribute to the observed increase in membrane fluidity in activated T-lymphocytes. Furthermore, in rat lymphocytes supplementation of the culture medium with PUFA lead to increases in membrane fluidity above that caused by mitogen stimulation alone and it was proposed that this change in fluidity might be the mechanism through which PUFAs inhibit lymphocytes responses (49).

PUFA acyl chains are highly disordered compared with saturated or monounsaturated acyl chains and therefore have unique structural effects on membranes (200). PUFAs can alter numerous properties of cell membranes, ultimately influencing protein function and signal transduction (201). One membrane property influenced by n-3 PUFAs is the organisation of membrane subdomains called lipid rafts. Lipid rafts are rich in sphingolipids and cholesterol and, relative to the surrounding membrane, are generally enriched in phospholipids containing saturated acyl chains (202). This allows close packing of lipids within rafts since the acyl chains of sphingolipids are also saturated and because saturated fatty acids are able to pack tightly with cholesterol (203). It is proposed that tight packing of lipids in lipid rafts is responsible for their resistance to extraction with non-ionic detergents; a signature property of lipid rafts (202). Many proteins important in signal transduction are predominately found in lipid rafts and consequently lipid rafts are thought to organise particular proteins to optimise their signalling capacity (202). Several important processes are reported to involve lipid rafts including T-lymphocyte activation and signal transduction (204-206). n-3 PUFAs have been reported to disrupt the composition and organisation of lipid rafts in T-lymphocytes and in turn suppress their activation (204, 207-209). Consequently, it has been suggested that a lipid raft mediated mechanism may contribute to the effects of n-3 PUFA on immune cell function; in other words, the immunomodulatory effects of n-3 PUFAs have been linked to their ability to modify lipid rafts (209, 210).
1.10.6 Evidence from the delta-6 desaturase knock-out mouse

A recent study used the delta-6 desaturase knock-out (D6KO) mouse model to study the immunomodulatory effects of individual n-3 and n-6 PUFA (211). The D6KO mouse lacks the Fads2 gene that encodes the delta-6 desaturase protein, thereby blocking the rate-limiting step in n-3 and n-6 PUFA metabolism. One of the most striking characteristics of this mouse model is sterility (212). Importantly, the D6KO mouse allows determination of the isolated effects of individual PUFAs on cell and tissue function, as it avoids the confounding effect of conversion to longer chain derivatives, and also provides a way to assess the importance of conversion of 18:2n-6 and 18:3n-3 to longer chain n-6 and n-3 PUFAs, respectively. D6KO mice fed diets containing the n-6 LC PUFA precursor 18:2n-6 displayed a lower proportion of unchallenged splenic CD4+ T-lymphocytes compared to wild-type mice (211). Furthermore, again in mice fed 18:2n-6 enriched diets, IFN-γ production in LPS stimulated splenic mononuclear cells was 5-times lower in D6KO compared to wild-type mice. Both of these effects were rescued by dietary repletion with 20:4n-6, a downstream conversion product of 18:2n-6. These results suggest that these properties are dependent, to some extent on the conversion of 18:2n-6 to 20:4n-6, when dietary sources of 20:4n-6 are lacking. 20:4n-6 was more potent than 18:2n-6 in promoting IFN-γ production and other pro-inflammatory cytokines (TNF-α and IL-6). There were no differences in IFN-γ production between mice fed 18:3n-3 or 20:5n-3/22:6n-3 based diets, which explains why there were no differences in IFN-γ production between D6KO and wild-type mice fed an 18:3n-3 enriched diet. Feeding 20:5n-3/22:6n-3 compared to 18:3n-3 significantly increased IL-10 production but there was no effect of genotype on production of this cytokine in mice fed 18:3n-3, indicating that functioning conversion was not able to emulate the effects of dietary supplementation. However, feeding 18:3n-3 did increase IL-10 production compared to 18:2n-6 and 20:4n-6 based diets, suggesting a possible independent effect of 18:3n-3. The amounts of n-6 and n-3 PUFAs fed to the mice in this study exceeded typical dietary intakes when translated to humans, a limitation highlighted by the authors. Furthermore, this study was limited by the number of immune functions assessed and did not analyse lymphocyte proliferation, arguably the most well-characterised immunomodulatory effect exerted by n-3 PUFAs. The results from this paper suggest that newly synthesised PUFAs serve important immunomodulatory roles when exogenous sources of conversion products are lacking but a more extensive analysis is required.

1.11 Epigenetic mechanisms

Studies have linked the synthesis or status of n-3 and n-6 PUFA with the mRNA expression level of the genes encoding the desaturase and elongase enzymes that function in the conversion pathway (104, 105, 213). A possible mechanism emerging for the control of PUFA synthesis is epigenetic
regulation of these genes. Epigenetics is a term used to describe a number of different processes that can cause heritable changes in the expression of a gene without changes to the underlying DNA coding sequence. Epigenetic regulation occurs via three main mechanisms: DNA methylation, histone modifications and non-coding RNAs. Histone modifications and non-coding RNAs will be introduced first and then DNA methylation, which is perhaps the most well characterised form of epigenetic regulation, will be discussed last.

1.11.1 Histone modifications

Histones are basic proteins that package and organise DNA into nucleosomes. Nucleosomes form the basic repeating unit of eukaryotic chromatin and consist of a segment of DNA, approximately 147 base pairs (bp), wound nearly twice around a histone octamer composed of two copies of each histone protein H2A, H2B, H3 and H4. Histone proteins contain two domains: a globular domain and an N-terminal tail domain. The N-terminal tails are exposed on the surface of the nucleosome and can therefore be subjected to modifications including acetylation, methylation, phosphorylation and ubiquitination (214). Histone modifications regulate gene expression by altering the accessibility of DNA resulting in regions of euchromatin, which is less condensed and transcriptionally active and heterochromatin, which is condensed and transcriptionally silent. Therefore, certain histone modifications promote transcriptional activation where others promote transcriptional repression. Histone acetylation, catalysed by histone acetyltransferases (HATs) is generally associated with increased gene expression (215). Histone acetylation involves addition of a negatively charged acetyl group to lysine residues of histone proteins, thereby reducing the affinity between histones and the negatively charged DNA. This results in a more open chromatin structure that is accessible for transcription. Histone acetylation can be reversed by the action of histone deacetylases (HDACs), resulting in a more condensed chromatin structure that is not accessible for transcription. Histone methyltransferases (HMTs) catalyse histone methylation. Histone methylation has varying effects on transcription depending on the specific lysine (K) or arginine (R) modified. Mono-, di- or tri-methylation of K9 on histone H3 (H3K9) or K20 on histone H4 (H4K20) are generally associated with transcriptional repression (216). In contrast, methylation of K4 on histone H3 (H3K4) and R3 on histone H4 (H4R3) are associated with active transcription (217, 218). Recent discoveries have demonstrated that histone methylation is reversible, which occurs by removal of methyl groups by histone demethylases (219).

1.11.2 Non-coding RNAs

Non-coding RNAs (ncRNAs) function as RNA molecules and are not translated into protein. They can act to regulate gene expression at the transcriptional and post-transcriptional level and
therefore can influence a large variety of biological functions. The ncRNAs involved in epigenetic processes can be categorised into two main groups according to the length of the transcript: short ncRNAs (<30 nucleotides) and the long ncRNAs (>200 nts). There are three major classes of short ncRNAs: microRNAs (miRNAs), short interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). MiRNAs and SiRNAs function in a similar way, causing gene silencing through complementary base pairing with target mRNAs, leading to degradation or translational repression (220). More recently, evidence indicates that siRNAs and miRNAs can guide chromatin remodelling in mammalian cells and therefore bring about transcriptional gene silencing (221, 222). siRNAs have been reported to guide transcriptional silencing complexes to specific chromosomal locations, promoting H3K9 methylation and subsequent chromatin condensation (223, 224). The most thoroughly understood function of piRNAs is the repression of transposable elements through complementary base pairing and target slicing, particularly in germ line cells. A role for piRNAs in transcriptional gene silencing through heterochromatin formation is also emerging (225). Finally, long ncRNAs play an essential role in genomic imprinting and X-chromosome inactivation (226). The gene silencing effects of long ncRNAs appear to involve recruitment of histone modifying complexes such as the polycomb repressive complex 2 (PRC2), which induces H3K27 methylation (227, 228).

1.11.3 DNA methylation

DNA methylation is a stable epigenetic mark that can be transmitted through DNA replication and cell division (229). In mammals nearly all DNA methylation occurs on cytosine residues of CpG dinucleotides (230). CpG sites are regions of DNA where a cytosine nucleotide occurs adjacent to a guanine nucleotide and the ‘P’ stands for the intervening phosphate group. DNA methylation involves the transfer of a methyl group from S-adenosyl methionine (SAM) to the 5 position of cytosine to form 5-methyl cytosine and is catalysed by DNA methyltransferases (DNMTs). CpG dinucleotides are not randomly distributed throughout the genome but are clustered at the 5 prime (5’) ends of genes in regions known as CpG islands (231). CpG islands have been defined as a region greater than 200 base pairs with an observed ratio for the occurrence of CpGs of greater than 0.6 (232). CpG islands are found in the promoters of approximately 60% of all mammalian genes (233). In general, high methylation of CpG islands is associated with transcription repression and low methylation is associated with activated gene expression (234). However, this is an oversimplification as there is increasing evidence to suggest that the binding of some transcription factors is enhanced by DNA methylation (235-237) and methylation can also act to block the binding of a repressive transcription factor (238) and therefore upregulate transcription.

The chemical modification brought about by DNA methylation results in altered DNA-protein interactions, which can result in either direct or indirect blocking of transcription. Direct blocking
involves the methyl group physically preventing the binding of transcriptional machinery such as RNA polymerase II and transcription factors to their recognition sequence (239). DNA methylation can indirectly repress gene expression by the recruitment of proteins that bind to methylated cytosines, such as methyl cytosine binding protein 2 (MeCP2) (240, 241). Once, bound MeCP2 recruits a corepressor complex containing histone deacetylases (HDACs) and histone methyltransferases (HMTs) (242, 243). Deacetylation and methylation of histone tails leads to a more condensed chromatin structure, making the transcription initiation site inaccessible to the transcription machinery (244, 245). MeCP2 therefore acts to link the epigenetic processes of DNA methylation and histone modifications (Figure 1.5).

Figure 1.5. Schematic diagram of epigenetic silencing of transcription.
Low DNA methylation is associated with increased gene transcription level through binding of transcription factors (TF) and RNA polymerase to the promoter region. Methylation of CpG sites represses gene transcription by preventing binding of TFs and RNA polymerase and via recruitment of MeCP2. MeCP2 recruits a corepressor complex containing histone deacetylases (HDACs) and histone methyltransferases (HMTs), which induces an inactive chromatin state.

DNA methylation is critical for mammalian development. It is responsible for ensuring silencing of genes on the inactive X chromosome in females, maintenance of genomic imprinting where either the maternal or paternal allele is switched off and long term silencing of non-coding DNA (246). Genome wide demethylation occurs twice during mammalian development. Demethylation and de novo methylation occurs during gametogenesis and plays a critical role in the establishment of parental-specific methylation marks in imprinted genes (247). Extensive demethylation (except in
imprinted genes) also occurs between fertilisation and preimplantation development and is followed by de novo methylation of the embryo genome by DNMT3a and DNMT3b (248-250). DNA Methylation patterns are conserved after replication by the maintenance methyltransferase DNMT1. DNMT1 recognises hemi-methylated DNA and catalyses transfer of methyl groups to cytosine nucleotides on the unmethylated daughter strand thereby maintaining methylation patterns through cell division (250). DNA methylation and other epigenetic mechanisms are essential for cell differentiation during development.

Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. However, recent evidence indicates that plasticity in DNA methylation does not stop at birth and instead can change throughout the life course (251). For example, a progressive decrease in global DNA methylation has been reported with increasing age. In humans a progressive loss of methylation in genome wide repetitive elements was reported with increasing age in subjects aged between 55 and 92 years (252). In a different study, total genomic DNA methylation was found to be significantly lower in older (aged 65 to 80 years) than younger (aged 20 to 30 years) individuals (253). In contrast, increased methylation has been reported at specific gene promoters with increasing age (254, 255). These age-related methylation changes are similar to those seen in cancer cells. Cancer progression is characterised by a decrease in global methylation (256), which affects chromosomal stability (257). Cancer is also associated with hypermethylation of specific gene promoters, particularly of tumour suppressive genes (258).

In addition to the effects of aging and disease, recent evidence indicates that epigenetic mechanisms can be modified by environmental factors, such as nutrition and environmental pollutants, both during development and later in the life course (251). Of particular relevance, dietary factors have been demonstrated to affect the methylation status of individual CpG dinucleotides in the FADS2 promoter, in studies in rodents (213, 259, 260) and human PBMCs (261). Furthermore, these studies reported that increased DNA methylation of individual CpG dinucleotides in the FADS2 promoter was associated with decreased FADS2 mRNA expression. The findings from the above-mentioned studies are discussed in more detail in section 5.1 in Chapter 5.

Finally, regulation of gene expression by DNA methylation has been shown to play a central role in immune responses. Specifically, DNA methylation changes are important for the differentiation of Th-lymphocytes into distinct effector subsets, which is based on the types of cytokines produced (262). Certain histone modifications have also been reported to be associated with Th-lymphocyte differentiation (263, 264). Again, epigenetic regulation of Th-lymphocyte differentiation is discussed in more detail in section 5.1 in Chapter 5.
1.12 Rationale, aims and hypotheses

Long chain n-3 PUFAs, particularly 20:5n-3 and 22:6n-3 are reported to have many beneficial effects on human health. In particular, findings from certain studies suggest they may have important effects on specific immune cell functions, although findings are not consistent in all studies. An early study demonstrated that PUFA synthesis is activated in human T-lymphocytes that have been stimulated to proliferate. However, this study was conducted in a small number of volunteers and there has since been no further investigation into the activity or regulation of PUFA synthesis in proliferating T-lymphocytes (see aim 1). Studies have demonstrated that the activity of the PUFA synthesis pathway is related to the mRNA expression levels of the genes encoding the desaturase and elongase enzymes that operate in the pathway (see aim 2). Furthermore, FADS2 transcription has been shown to be related to the DNA methylation status of specific CpG dinucleotides in its promoter region and DNA methylation is also known to be important for regulating the expression of specific genes involved in T-lymphocyte differentiation following activation (see aim 3).

A high level of PUFA synthesis has been reported in a spontaneously dividing T-lymphocyte leukaemic cell line (Jurkat), resembling the wider dysregulation of de novo fatty acid synthesis seen in cancer cells. However, there has been no direct comparison of PUFA synthesis in Jurkat cells and primary T-lymphocytes. Furthermore, it is not known whether the high level of PUFA synthesis is mediated at the transcriptional level nor whether it is important for supporting cellular proliferation (see aims 4 to 7).

Finally, a number of studies have reported gender differences in the conversion of 18:3n-3 to longer chain n-3 PUFAs in humans. However, this has not been investigated across the life course or specifically in immune cells. The effect of age on the activity of the PUFA synthesis pathway is less clear and requires further investigation (see aim 8).

The general aims and hypotheses of this thesis, related to the activity and regulation of the PUFA biosynthesis in humans are given below with more specific versions given in the individual results chapters.

Aims

1. To compare the activity of the PUFA synthesis pathway in unstimulated and mitogen-stimulated PBMC populations.
2. To compare the mRNA expression levels of the genes encoding the enzymes that function in the PUFA biosynthesis pathway in unstimulated and mitogen-stimulated PBMC populations.
3. To determine whether any changes in mRNA expression in unstimulated and mitogen-stimulated PBMC populations are associated with DNA methylation changes.
4. To compare the activity of the PUFA synthesis pathway in mitogen-stimulated PBMC populations and Jurkat cells.

5. To compare the mRNA expression levels of the genes encoding the enzymes that function in the PUFA biosynthesis pathway in mitogen-stimulated PBMC populations and Jurkat cells.

6. To determine whether any differences in mRNA expression between mitogen-stimulated PBMC populations and Jurkat cells are associated with DNA methylation changes.

7. To investigate whether inhibition of the PUFA synthesis pathway affects the proliferation of mitogen-stimulated lymphocytes and Jurkat cells.

8. To determine the effect of age and gender on the activity of the PUFA synthesis pathway and the mRNA expression levels of the genes encoding the enzymes that function in the pathway, in mitogen-stimulated PBMC populations.

**Hypotheses**

1. The activity of the PUFA synthesis pathway is higher in mitogen-stimulated compared to unstimulated PBMCs.

2. The mRNA expression levels of the genes encoding the pathway enzymes are higher in mitogen-stimulated compared to unstimulated PBMCs.

3. DNA methylation levels in the promoter regions of the genes encoding the pathway enzymes are lower in mitogen-stimulated compared to unstimulated PBMCs.

4. The activity of the PUFA synthesis pathway is higher in Jurkat cells compared to mitogen-stimulated PBMCs.

5. The mRNA expression levels of the genes encoding the pathway enzymes are higher in Jurkat cells compared to mitogen-stimulated PBMCs.

6. DNA methylation levels in the promoter regions of the genes encoding the pathway enzymes are lower in Jurkat cells compared to mitogen-stimulated PBMCs.

7. Inhibition of the PUFA synthesis pathway decreases proliferation of mitogen-stimulated lymphocytes and Jurkat cells.

8. The activity of the PUFA synthesis pathway and mRNA expression levels of the pathway enzymes are higher in females compared to males and decrease with increasing age in mitogen-stimulated PBMCs.
Chapter 2: Methods
2.1 Reagents

A list of reagents, chemicals, kits and suppliers can be found in Appendix A. All water used was generated using a Barnstead NANOpure Diamond™ (Thermo Scientific, UK) reagent grade water purification system unless otherwise stated.

2.2 Human study protocol

Healthy volunteers were recruited to take part in the ‘Epigenetic regulation of omega-3 polyunsaturated fatty acid synthesis (EPUFA)’ research study conducted at the Centre for Endocrinology and Diabetic Research (CEDAR) at the Royal Surrey County Hospital (RSCH).

2.2.1 Ethical approval

Before the EPUFA study was undertaken approval was sought from two ethical review bodies: the NHS Research Ethics Service (NRES) and the University of Surrey ethics committee (UEC). For NRES review an application was prepared using the Integrated Research Application System (IRAS) and submitted to the NRES committee North West – Preston. The study was reviewed and given a favourable ethical opinion by both the NRES research ethics committee (REC) North West – Preston (REC reference 14/NW/1048) and the University of Surrey ethics committee (UEC reference EC/2014/112/FHMS). Clinical governance was provided by the RSCH NHS Foundation Trust Research and Development Office.

2.2.2 Inclusion and exclusion criteria

Healthy, White Caucasian, males and females aged between 18 and 71 with a BMI between 18.5 kg/m² and 30 kg/m² were recruited to take part in the research study. The exclusion criteria are listed below.

Exclusion criteria:
- Aged <18 or >71 years
- BMI <18.5 kg/m² or >30 kg/m²
- Pregnant women
- Women receiving hormone based contraceptives
- Women receiving hormone replacement therapy
- Diagnosed type 1 or type 2 diabetes
• Use of any prescribed medication known to affect lipid metabolism (any medications listed by potential volunteers were passed on to the named clinician for the study to confirm acceptability)
• Smokers
• Male consuming more than 28 units of alcohol per week or females consuming more than 21 units of alcohol per week
• Consumption of more than one portion of oily fish per week
• Consumption of fish oil capsules
• Participation in another research study

Participants were not asked whether they were taking any non-prescribed medications and therefore individuals taking anti-inflammatory agents such as aspirin were potentially included in the study. This represents a limitation since aspirin affects the conversion of n-3 PUFAs into downstream inflammatory mediators such as resolvins (See section 1.10.2 and Figure 1.4).

2.2.3 Sample size calculations

Preliminary power analysis showed that 10 males and 10 females would be sufficient to detect a 2-fold difference in synthesis of [1-13C]20:5n-3 from [1-13C]18:3n-3 between males and females with 80% power and P < 0.05, based on data from Burdge and Wootton, 2002 (99), and Burdge et al., 2002 (100). Therefore, the study aimed to recruit 10 males and 10 females into each of the following age groups to ensure an even spread of ages: 18 to 37, 38 to 54 and 55 to 71 years.

2.2.4 Recruitment

Participants were recruited via posters and emails. Individuals interested in participating were emailed an information sheet (Appendix B.1) and were asked to complete a screening questionnaire (Appendix B.2). Potential participants that appeared to meet the inclusion criteria were invited to attend a study appointment at the CEDAR. A total of 89 volunteers attended the study appointment and of these volunteers three did not meet the inclusion criteria so a total of 86 volunteers were included in the study. Samples from 18 volunteers were used to optimise the experimental techniques leaving 68 volunteers (31 males and 37 females) in the main study.

2.2.5 Study appointment

Participants were required to arrive fasted, having had nothing to eat or drink except water in the previous 10 h. Written informed consent was obtained (Appendix B.3) before the participants’ height, weight and percentage body fat were measured to confirm eligibility. Height was measured
using a stadiometer with shoes removed. Weight and body fat composition measurements were made using a TANITA bioelectrical impedance device.

Two individual cotton swabs (Fisher Scientific) were used to collect buccal cells from each side of the mouth. Participants were asked to brush the inside of their cheeks with the swabs, which were then immersed in the collection tube containing 3 ml of water, sealed and placed on ice until processing within 2 h.

Blood pressure was measured in triplicate by a research nurse who then collected blood samples with a winged blood collection needle and vacutainer tube (BD Biosciences). Lithium heparin tubes were used to collect blood (42 ml) for peripheral blood mononuclear cell (PBMC) isolation, potassium ethylenediaminetetraacetic acid (K$_2$-EDTA) tubes (4 ml) for total haemoglobin and plasma triglycerides (TG), total cholesterol and high density lipoprotein (HDL) cholesterol analysis and sodium fluoride/potassium oxalate tubes (2 ml) for plasma glucose analysis. Blood samples in K$_2$-EDTA and sodium fluoride/potassium oxalate tubes were stored on ice until processing within 2 h. The lithium heparin blood samples were stored in an air and watertight container, which was placed in a sample transport box cooled with two ice packs and transported to the University of Southampton where the blood samples were processed within 6 h from time of collection. An overview of the study day is given in Figure 2.2.

### 2.3 Blood biochemistry analyses

Total haemoglobin and plasma TG, total cholesterol, HDL cholesterol and glucose were measured in order to characterise the EPUFA study population. The results of the blood biochemistry analyses were sent to each participant’s general practitioner (GP) and a copy was sent to the volunteer.

Total haemoglobin was measured using a GEM Oxygenation portable laboratory (OPL) portable analyser (Werfen, Spain) according to manufacturer’s instructions at the point of sampling. Blood for metabolites was centrifuged at 500 x g for 15 min at 4°C and the plasma was stored at -80 °C until analysis. Assays for measurement of plasma TG, HDL-cholesterol, total cholesterol and glucose were carried out on a COBAS MIRA auto-analyser (Roche Diagnostic, USA) using the following Horiba ABX Pentra kits: Triglycerides CP (kit A11A01668), HDL Direct CP (kit A11A01636), Cholesterol CP (A11A01634) and Glucose PAP CP (kit A11A01668). All assays were carried out according to manufacturer’s instructions and low and high quality controls (Horiba N and P controls) were included at the beginning, middle and end of each run in each run.
2.4 Isolation of PBMCs from heparinised blood samples

PBMCs were isolated from 40 ml of the heparinised blood by density gradient centrifugation using an adaptation of the method described in Lomax et al., 2012 (265). A 10 ml volume of the density gradient solution Histopaque®-1077 (Sigma-Aldrich) was aliquoted into each of four sterile 50 ml centrifuge tubes and equilibrated to RT. An equal volume of blood was layered slowly over the Histopaque®-1077 ensuring two distinct layers were visible. Samples were centrifuged at 845 x g for 15 min at RT with no break applied. During centrifugation, differential migration of blood cells causes layers of different cell types to form (Figure 2.1). The bottom layer contains erythrocytes. Immediately above this in the Histopaque®-1077 layer there are mostly granulocytes. PBMCs are found at the interface between the Histopaque®-1077 layer and the plasma (top layer) in a region called the buffy coat. Platelets, also found in the buffy coat, are removed by subsequent washing and centrifugation steps. The samples were carefully removed from the centrifuge ensuring no disruption of layers.

Plasma collection:

The plasma layer was transferred into a new sterile 50 ml centrifuge tube. Next 200 µl of plasma was transferred to a screw capped glass tube and 600 µl 0.9% (w/v) NaCl was added ready for subsequent total lipid extraction (described in section 2.12.1). A further 2 ml of plasma was aliquoted into each of two 2 ml tubes and stored at -80°C. The remaining plasma was kept at 4°C until required.

PBMC collection and handling for downstream analyses:

The layer containing the PBMCs was transferred into a sterile 50 ml sterile centrifuge tube and RPMI-1640 medium (Sigma-Aldrich) was added to a total volume of 40 ml in order to wash the cells before centrifuging at 300 x g, for 10 min at RT. After centrifugation the RPMI-1640 was decanted from the PBMC pellet and the cells were resuspended in 1 ml RPMI-1640. 10 µl of the cell suspension was removed and diluted with 90 µl RPMI-1640, which was then diluted a further 10 times with 0.4 % trypan blue solution (Sigma-Aldrich) before counting using a haemocytometer.

The concentration of cells was adjusted to 2 x 10⁶/ml with RPMI-1640. A total of 10 x 10⁶ cells were transferred into a 15 ml centrifuge tube (these cells were designated for analysis of cell phenotypes using flow cytometry). A calculated volume of autologous plasma was then added to the remaining cells in the 50 ml centrifuge tube (these cells were designated for cell culture) to achieve a concentration of 2% autologous plasma in RPMI-1640. Both tubes were centrifuged at 300 x g, for 10 min at RT.
Cells designated for analysis by flow cytometry:

The cells designated for flow cytometry were washed with 10 ml phosphate buffered saline (PBS; Sigma-Aldrich) and centrifuged as before. The cell pellet was resuspended in 1 ml PBS before counting using a 10-fold dilution in trypan blue and a haemocytometer. A total of $5 \times 10^5$ cells were transferred into each of four 5 ml round bottom tubes and stored at 4°C ready for analysis of cell phenotypes. Where there were surplus cells, $2 \times 10^6$ cells were transferred into 0.5 ml micro-centrifuge tubes and pelleted by centrifugation at 300 x g, for 10 min at RT. The PBS wash was removed and the pellet was snap frozen on dry ice before being transferred to -80°C storage. These tubes were used for pre-culture mRNA expression and DNA methylation measurements.

Cells designated for cell culture:

The cells designated for cell culture were washed with 10 ml RPMI-1640 containing 2% autologous plasma and centrifuged as before. The supernatant was removed and the cells were resuspended in 1 ml PBMC medium (see Appendix A.2.1 for composition) containing 5% (v/v) autologous plasma before diluting a 10 µl aliquot 10-fold in trypan blue and counting cells using a haemocytometer. Cells were adjusted to a concentration of $1 \times 10^6$ cells/ml and cultured as described in section 2.6.

Erythrocyte collection:

The erythrocyte layer from the original gradient centrifugation was transferred into a sterile 50 ml centrifuge tube and washed three times with two volumes of 0.9 % NaCl to one volume of erythrocytes and centrifuged between washes at 200 x g for 10 min at RT to pellet the cells. After the final wash, 500 µl of erythrocytes were transferred to a screw capped glass tube and 500 µl of 0.9% NaCl added ready for total lipid extraction (described in section 2.12.1). A further 1 ml of erythrocytes were added to each of two 2 ml tubes and stored at -80°C.

Figure 2.1. Schematic of PBMC isolation procedure.
Figure 2.2. Overview of the EPUFA study day.

The study day involved three locations: 1. Volunteers attended an appointment at the Centre for Endocrinology and Diabetic Research (CEDAR) at the Royal Surrey County Hospital (RSCH); 2. A portion of the blood samples were processed at the University of Surrey. 3. The remaining blood samples were transported to the University of Southampton where PBMCs were isolated and cultured.
2.5 Analysis of PBMCs using flow cytometry

The proportions of T-lymphocytes, B-lymphocytes and monocytes in the PBMC populations were determined by analysing the expression of the cell surface markers CD3, CD19 and CD14, respectively, using flow cytometry.

Flow cytometry was performed using a Becton Dickinson FACS Calibur flow cytometer equipped with a 488 nm laser. In flow cytometry, the microfluidic system of the flow cytometer enables single cells to pass through a laser beam and the light scattered from the cells is detected. A detector in front of the laser beam measures the forward scatter (FSC) and detectors to the side of the laser beam measure the side scatter (SSC). FSC is proportional to cell size and SSC to cell granularity and therefore different cell populations can be distinguished based on their light scattering characteristics. Fluorescence detectors measure the fluorescence emitted from stained cells. Data was acquired using CellQuest Pro analysis software (BD Biosciences, USA) and then exported to FlowJo software version 7.6.5 (TreeStar, Inc, USA) for further analysis.

2.5.1 Staining PBMCs with fluorescently conjugated antibodies

Staining was performed according to manufacturer’s instructions for each fluorescently conjugated antibody. A total of 5 x 10^5 PBMCs were added to each of four individual 5 ml round bottom tubes in 100 µl PBS. They were then incubated for 30 min at 4°C in the dark with 10 µl of the following mouse anti-human fluorescently conjugated monoclonal antibodies (Bio-Rad): anti-CD3 FITC (fluorescein isothiocyanate), anti-CD19 FITC, anti-CD14 RPE (R. Phycoerythrin) and IgG1 (FITC) isotype control (BD Biosciences) or no antibody as a no stain (negative) control. Anti-CD3 FITC and anti-CD14 RPE were added to the same tube as a dual stain. Anti-CD3 FITC and CD19 FITC were diluted 1 in 10 with water immediately prior to being added to the cells. After incubation the cells were washed with 2 ml cell wash solution (BD Biosciences) and centrifuged at 211 x g for 5 min. The cells were resuspended in 200 µl cell fix solution (BD Biosciences) and kept at 4°C until analysis within 48 h.

2.5.2 Optimisation of instrument settings for flow cytometry

Before data were collected the instrument settings were optimised using CellQuest Pro analysis software. First the forward scatter (FSC) and side scatter (SSC) detectors were adjusted to obtain a clear population of PBMCs in an appropriate location on the plot. Next, the FSC threshold was adjusted to exclude most of the debris without excluding any of the population of interest. A gate was then drawn around the PBMC population (Figure 2.3) and the fluorescence channel 1 (FL-1) and fluorescence channel 2 (FL-2) were adjusted to appropriately position the negative population.
and allow visualisation of the positive population in this region. Fluorochrome FITC was detected in FL-1 and RPE in FL-2. Finally, the fluorescence compensation was adjusted to correct for signal overspill between FL-1 and FL-2. The instrument settings (Appendix A.3) were then saved and used to analyse each sample. In all cases $10^4$ total events were collected for analysis.

2.5.3 Cell phenotyping data analysis

A gate was drawn around the total PBMC population for freshly isolated (pre-culture) PBMCs (Figure 2.3; R1). Following culture (post-culture) a gate was drawn to capture total PBMCs including activated lymphocytes (Figure 2.3; R2). Figure 2.3 shows that the monocyte population largely disappeared following culture. The events within the PBMC gate (R1 or R2) were represented in a histogram showing fluorescence intensity on the x-axis and the number of events (cell count) on the y-axis Figure 2.4. The no stain control samples were used to set the position of a marker (M1) over positively staining events. The isotype control was used to verify that there was no non-specific binding. The percentage of events falling within the boundaries of M1 were reported as the percentage of events positive for the measured cell surface marker and therefore corresponding cell type within the PBMC population.

![Figure 2.3. Gating PBMC populations using flow cytometry.](image)

FSC and SSC settings were adjusted to obtain a clear population of PBMCs. The graphs show a PBMC population prior to culture (pre-culture), after 48 h culture without activation by Con A (No Con A) and after 48 h culture with activation by 5 µg/ml Con A. A gate (R1 for pre-culture and R2 for post-culture) was drawn around the cells so only the PBMC population would be used for subsequent analysis.
Figure 2.4. Histograms showing expression of specific cell surface markers in PBMCs. (A) The cell surface marker CD3 is specific to T-lymphocytes, (B) CD19 to B-lymphocytes and (C) CD14 to monocytes. Red line shows the no stain control and the blue line shows the stained sample with the markers M1 and M2 set to include positively staining cells.

2.6 Culture of PBMCs isolated from lithium heparin blood

PBMCs isolated as described in section 2.4 were cultured in 52cm² tissue culture dishes at 1 x 10⁶ cells/ml in PBMC medium (see Appendix A.2.1 for composition) supplemented with 5% autologous plasma, 20 µM stable isotope labelled 18:3n-3 ([1-¹³C]18:3n-3) and 5 µg/ml concanavalin A (Con A). Details of the preparation of the stock solutions of [1-¹³C]18:3n-3 and Con A can be found in Appendix A.2.2. For 34 volunteers of the total 68 volunteers, parallel cultures were also prepared, without addition of Con A. Before adding to cells, a 0.8 ml aliquot of the supplemented medium was transferred to a screw cap glass tube ready for total lipid extraction (section 2.12.1). Cells were cultured for 48 h at 37°C under 5% CO₂ in a humidified atmosphere.

2.6.1 Harvesting PBMCs for downstream analyses

After 48 h the PBMCs were collected from dishes using a cell scraper to remove any adhered cells and the cell suspensions were transferred to 15 ml centrifuge tubes and centrifuged at 300 x g for 10 min. Each cell pellet was washed once with ice cold PBS, centrifuged as before and then resuspended in 1 ml ice cold PBS for cell counting. A 10 µl aliquot of cell suspension was diluted 10-fold with PBS and then 2-fold with 0.4% trypan blue solution and counted using a haemocytometer.

1 x 10⁶ cells were transferred to each of four 1.5 ml microcentrifuge tubes and centrifuged at 300 x g for 10 min. The supernatants were removed and the cell pellets were snap frozen on dry ice prior to being transferred to storage at -80°C for subsequent RNA and DNA extraction (described in section 2.14). 5 x 10⁵ cells were transferred to three individual 5 ml round bottom tubes in a final
volume of 100 µl PBS and stored at 4°C ready for measurement of cell activation by flow cytometry (described in section 2.7). The remaining cells were centrifuged at 300 x g and resuspended in 0.8 ml 0.9% (w/v) NaCl. A 20 µl aliquot of the cell suspension was transferred to a 0.5 ml microcentrifuge tube and frozen on dry ice before storage at -80 °C for later total cell protein quantification (see section 2.13). Total lipid extracts and fatty acid methyl esters (FAMES) were prepared from the remaining cells as described in section 2.12.

2.7 Measurement of T-lymphocyte activation by CD69 expression

The proportion of activated T-lymphocytes was determined by measuring the cell surface expression of the activation marker CD69 on events in the PBMC region using flow cytometry. Following cell culture 5 x 10⁵ PBMCs suspended in 100 µl PBS were incubated with 10 µl mouse anti-human CD69 FITC monoclonal antibody for 30 min at 4°C in the dark and processed for analysis by flow cytometry as described in section 2.5, using the same instrument settings. A gate (R2) was drawn around the PBMC population (Figure 2.3, post culture). The events within R2 were represented in a histogram and a marker (M1) was drawn over events staining positively for CD69 FITC (Figure 2.5). The proportion of events falling within the boundaries of M1 were reported as percentage of cells expressing CD69.

Figure 2.5. Histograms showing CD69 expression in PBMCs.  
The red line shows the isotype control population and the blue line shows the CD69 (FITC) stained samples within the PBMC gate (R2 – see Figure 2.3) with the marker M1 set to include positively staining cells. The graphs show cells cultured for 48 h in the absence of Con A (no Con A) and cells cultured for 48 h in the presence of 5 µg/ml Con A (+ConA).
2.8 Revival and cell culture of purchased cryopreserved PBMCs

Cryopreserved PBMCs purchased from StemCell Technologies (Canada) were used for additional cell culture experiments. Purchased PBMCs were used to determine the effect of 18:3n-3 concentration on PBMC viability (section 2.9) and proliferation (section 2.10) as part of initial optimisation experiments before commencement of the EPUFA study. Purchased PBMCs were also used in experiments to determine the effect of the delta-6 desaturase inhibitor (SC-26196) on proliferation. Purchased PBMCs were used as these experiments were conducted after completion of the EPUFA study, in order to investigate hypotheses generated from the study findings.

Purchased PBMCs were cultured in PBMC medium containing 10% (v/v) fetal bovine serum (FBS). The composition of the cell culture medium and details of cell culture treatment compounds are given in Appendix A.2. The PBMCs arrived frozen on dry ice and were transferred immediately to liquid nitrogen storage. When required, each vial of cryopreserved PBMCs was thawed in a 37°C water bath; being removed from the water bath when only a small frozen cell pellet remained. A 20 µl aliquot of cells was set aside for counting using 0.4% trypan blue solution and a haemocytometer to confirm adequate cell number and viability. The remaining cell suspension was transferred to a 15 ml centrifuge tube. The vial was rinsed with 1 ml of PBMC medium containing 10% FBS (equilibrated to 37°C) and added drop-wise to the cells whilst swirling the tube. A further 10 ml medium was added on top and the cell suspension centrifuged at 300 x g for 10 min at RT. The supernatant was carefully removed and the cells resuspended in 1 ml PBMC medium containing 10% FBS. Cells were counted and adjusted to a density of 1 x 10^6 cells/ml before culturing in 52 cm² tissue culture dishes at 37°C under 5% CO₂ in a humidified atmosphere.

2.9 Cell viability assays

The viability of purchased PBMCs, after exposure to specific cell culture treatments, was measured using two different viability assay kits: CellTiter-Glo Luminescent Cell Viability Assay (Promega) and LIVE/DEAD Fixable Red Dead Cell Stain Kit (Thermo Fisher Scientific). Both kits allowed rapid measurement of cell viability for large sample numbers.

2.9.1 CellTiter-Glo Luminescent Cell Viability Assay

This is a bioilluminescence assay based on quantitation of adenosine triphosphate (ATP) to determine cell viability since ATP is an indicator of metabolically active cells. The cells are lysed, releasing ATP and generating a luminescent signal that is proportional to the amount of ATP present.
In order to measure cell viability using this assay, 1 x 10^5 purchased PBMCs in 100 µl of PBMC medium containing 10% (v/v) FBS were plated in each well of opaque walled, flat bottom 96 well plates with six replicates for each treatment to be tested and vehicle control. Six replicate control wells, containing medium without cells, were also prepared in order to obtain a background luminescence value. The plate was then incubated at 37°C with 5% CO₂ in a humidified atmosphere for the required amount of time depending on the specific experiment. Details of the treatments and incubation times for specific experiments are given in Chapter 3 (section 3.2.4). The plate was then equilibrated to room temperature for 30 min and processed according to manufacturer’s instructions. Luminescence was measured on a VarioSkan Flash luminometer (Thermo Fisher Scientific). Values were expressed relative to the vehicle control, which was set at 100%.

### 2.9.2 LIVE/DEAD Fixable Red Dead Cell Stain Kit

This kit differentially stains live and dead cells, based on their membrane integrity, for analysis using flow cytometry. The version of the kit containing the red fluorescent reactive dye (excitation max 595 nm and emission max 615 nm) was chosen as it is suitable for dual measurement with CFSE (excitation max 490 nm and emission max 520 nm), where dual measurement was required for gating live cells for analysis of proliferation (see section 2.10). The fluorescent dye supplied with the kit reacts with cellular amines. For live cells, the dye cannot permeate the cell membrane and therefore only the cell surface amines are available to react with the dye. The membranes of necrotic cells become compromised allowing the dye to permeate and react with free amines inside the cell in addition to those on the cell surface. The result is much brighter staining for dead cells compared with live cells allowing clear discrimination.

#### 2.9.2.1 Staining PBMCs with LIVE/DEAD Fixable Red Dead Cell Stain

A total of 2 x 10^5 PBMCs per well were plated in clear flat bottom 96 wells plates in 200 µl of PBMC medium containing 10% (v/v) FBS. There were six replicates for each test compound, vehicle control and blank (media). An additional six replicates treated with 1 mM 22:6n-3 were included as a cell death control. After the culture period (see Chapter 3, section 3.2.4 for details) the cells were transferred into 5 ml round bottom tubes, washed with 0.5 ml PBS and centrifuged at 300 x g for 10 min. After centrifugation the PBS wash was removed and the PBMCs were resuspended in 200 µl PBS containing 1 µl/ml red reactive dye. The cell suspension was incubated at 4°C for 30 min, protected from light. The cells were then washed with 0.5 ml of PBS containing 1% (w/v) bovine serum albumin (BSA) and centrifuged at 300 x g for 10 min. The supernatant was discarded and the cell pellet was resuspended in 200 µl PBS with 1% BSA.
2.9.2.2 Analysis of cell viability using flow cytometry

The instruments settings used to acquire data are listed in Appendix A.3. A total of $10^4$ events were recorded and the viability dye was measured in FL-2. A histogram showing FSC on the x-axis and number of events on the y-axis was drawn using FlowJo analysis software and a marker (M1, Figure 2.6) was set to include dead and live cell populations but exclude debris. The events in M1 were used to draw another histogram with FL-2 fluorescence on the x-axis and number of events on the y-axis. The cell death control, which displayed two clear peaks of differing fluorescence intensity (Figure 2.6, graph C-bottom) was used to position a marker (M2) over the dead cell population. The percentage of events falling within the boundaries of M2 as a proportion of events in M1 were recorded as the percentage of non-viable cells.

![Image of histograms showing FSC against count for events with marker M1, and FL-2 fluorescence against count for events with marker M2 over non-viable cells.](image)

Figure 2.6. Measurement of PBMC viability using LIVE/DEAD Fixable Red Dead Cell Stain Kit. The top three histograms display FSC against count for all events with the marker M1 over total cells. The bottom three histograms display fluorescence in FL-2 against count for events in M1 with the marker M2 over the non-viable cells. (A) Negative control (unstained) PBMCs. (B) Stained PBMCs. (C) Positive control for cell death (PBMCs treated with 1 mM 22:6n-3).

2.10 Analysis of PBMC proliferation using carboxyfluorescein succinimidyl ester (CFSE)

CFSE is a dye that can be used for monitoring of cell divisions using a flow cytometer equipped with a blue 488 nm laser. CFSE passively diffuses across cell membranes where it is cleaved by
intracellular esterases making it highly fluorescent; nonviable cells cannot cleave the dye and remain non-fluorescent. The succinimidyl ester group enables CFSE to covalently bind to protein amine groups within cells and as they divide the dye is distributed equally between progeny cells resulting in a halving of the fluorescence of daughter cells.

Cryopreserved PBMCs were thawed as described in section 2.8 and then a total of $4 \times 10^7$ viable PBMCs were suspended in 1 ml PBS containing 5% (v/v) FBS ready for staining with CFSE as detailed below. A further $2 \times 10^6$ PBMCs were adjusted to $1 \times 10^6$ cells per ml in PBMC medium containing 10% (v/v) and were used as an unstained control.

### 2.10.1 PBMC treatment with CFSE

One vial containing 500 µg of lyophilised CFSE (Affymetrix eBioscience) was resuspended with 180 µl dimethylsulfoxide (DMSO) to make a 5 mM stock solution. CFSE was diluted directly into the 1 ml cell suspension containing approximately $4 \times 10^7$ viable PBMCs. After thorough mixing, the cells were incubated for 5 min at RT, protected from the light. CFSE staining was terminated by washing the cells with 10 ml PBS containing 5% (v/v) FBS and centrifuging at 300 x g for 5 min. The supernatant was discarded and the cell pellet was washed in the same manner twice more. After the final wash the cells were resuspended in 1 ml of PBMC medium containing 10% (v/v) FBS and counted using a haemocytometer. The concentration of viable cells was adjusted to $1 \times 10^6$/ml. 4 x $10^6$ cells were transferred to a fresh tube to be used as an unstimulated control. The remaining CFSE labelled cells and the unlabelled cells (set aside in section 2.10) were stimulated with Con A at a final concentration of 5 µg/ml. Stimulated CFSE labelled cells were then divided between three fresh tubes for treatment with SC-26196. Stock solutions containing 10 µM and 20 µM SC-26196 were prepared as described in Appendix A.2.2. Cells were then treated with 100 nM or 200 nM SC-26196 or just 0.02% (v/v) DMSO as a vehicle control. Cells were transferred to five individual 96 well flat bottom culture plates with eight replicates per plate for each SC-26196 treatment and vehicle control, two replicates for the CFSE stained but unstimulated cells and two replicates for the unstained cells. A further two replicates of CFSE stained stimulated cells treated with 1 mM 22:6n-3 were added to one plate as a positive control for cell death. Cells were maintained in a humidified cell culture incubator at 37°C in a 5% CO2 atmosphere for up to 144 h.

### 2.10.2 Preparation of CFSE labelled PBMCs for flow cytometry

The first plate was removed after 48 h for analysis and then one plate was removed every 24 h until 144 h. The remaining cells were transferred from the 96 well plates into 5 ml round bottom tubes, washed with 1 ml PBS and centrifuged at 300 x g for 10 min. The cell pellet was resuspended in 200
µl PBS containing 1 µl/ml of LIVE/DEAD red viability dye (see section 2.9.2 for details) and incubated at 4°C for 30 min, protected from light. Cells were washed with 1 ml PBS containing 1% (w/v) bovine serum albumin (BSA) and centrifuged for 5 min at 300 x g. The supernatant was discarded and the cells were resuspended in 200 µl 1% BSA (w/v) in PBS.

### 2.10.3 Measuring CFSE fluorescence using flow cytometry

CFSE fluorescence was measured in FL-1 and the LIVE/DEAD red viability dye fluorescence was detected in FL-2. The instruments settings were optimised for this analysis as described in section 2.5.2 and are detailed in Appendix A.3. A total of $10^4$ events were collected for all samples. The results were exported and analysed using FlowJo software version 7.6.5. The lymphocyte population was gated (R1) as shown Figure 2.7 (A) and non-viable cells were excluded from the analysis by combining R1 with a second gate (R2) around viable cells (Figure 2.7, B). The positive control for cell death was used to confirm the fluorescence intensity of the non-viable cells. The number of cell divisions for viable lymphocytes, shown as discrete peaks with decreasing CFSE fluorescence, were modelled and analysed using the FlowJo proliferation platform (Figure 2.7, C), typically set with a coefficient of variation (CV) of 5-10 and a peak ratio of 0.5. A root mean squared (RMS) of <10 was required for inclusion in the analysis. The following measures of proliferation were recorded:

- **Percent divided** – the percentage of cells from the original sample that divided.
- **Division Index** – the average number of divisions that a cell from the original population has undergone; this is the average number of divisions for the entire population including the undivided peak.
- **Proliferation Index** – the average number of cell divisions for the cells that entered cell division; this is the average number of division for the responding population.
**Figure 2.7. Analysis of proliferating lymphocytes stained with CFSE by flow cytometry.**

(A) A gate was drawn around lymphocytes based on their light scatter characteristics (R1). (B) Dead cells in R1 were excluded by introduction of a sub-gate (R2). (C) Histogram showing multiple CFSE fluorescence peaks of proliferating lymphocytes. The proliferation model applied by the FlowJo software is shown in pink. The CFSE fluorescence peak of undivided cells (peak zero) is indicated by a red line.

### 2.11 Cell line culture

#### 2.11.1 Revival and maintenance of HepG2 cells

In house stocks of HepG2 cells (European Collection of Authenticated Cell Cultures [ECACC]), were removed from liquid nitrogen storage and thawed in a 37°C water bath until only a small frozen cell pellet remained. The cell suspension was transferred to a 15 ml centrifuge tube, washed with 10 ml HepG2 medium (see Appendix A.2.1 for composition), centrifuged at 200 x g and resuspended in 1 ml HepG2 medium. Cells were counted using a haemocytometer before seeding in 75 cm² tissue culture flasks at a density of 30 x 10⁵ cells/cm² in 17 ml HepG2 medium. Once cultures reached approximately 80% confluency they were washed with 5 ml Hanks’ balanced salt solution (HBSS) before addition of 2 ml of 0.05% trypsin EDTA (Sigma-Aldrich). Cultures were then incubated at 37°C to detach cells from the bottom of the flask. After 2-5 min the cultures were removed from the incubator and 10 ml HepG2 media was added to the flask to neutralise the trypsin. The cells were then split equally between four flasks and cultured as before. HepG2 cells were always left to adhere for 24 hrs before any experimental treatments.

#### 2.11.2 Revival and maintenance of Jurkat cells

In-house stocks of Jurkat cells (ECACC), were removed from liquid nitrogen storage and thawed and washed as described for HepG2 cells but with Jurkat medium (see Appendix A.2.1 for composition) and with centrifuging at 300 x g. Cells were seeded in 25 cm² culture flasks at a density of 300 x 10⁵ cells/ml in 15 ml Jurkat medium. Cells were split between new flasks when density reached approximately 1 x 10⁶ cells/ml.
2.12 Fatty acid analysis

2.12.1 Total lipid extraction

Following cell culture, PBMC and Jurkat cells were pelleted by centrifugation and resuspended in 0.8 ml of 0.9 % (w/v) NaCl whereas HepG2 were scraped from the surface of the culture plate in 0.8 ml of 0.9 % (w/v) NaCl and transferred to a glass screw capped tube ready for downstream total lipid extraction. Plasma and erythrocytes were prepared for total lipid extraction as described in section 2.4. Total lipids were extracted using an adaptation of the method described by Folch (266).

A quality control (QC) sample (100 µg of L-α-phosphatidylcholine [Sigma-Aldrich]) and a blank were processed at the same time as the samples. The QC contained known proportions of 16:0 (33%), 18:0 (13%), 18:1n-9 (31%), and 18:2n-6 (15%), which were monitored for each run. Diheptadecanoyl-sn-glycero-3-phosphocholine (17:0 PC [Sigma-Aldrich]) was added to each sample and to the QC and blank as an internal standard for quantitative analysis. The amount added depended on the sample being processed (5 µg for PBMCs and 25 µg for Jurkat cells, HepG2 cells, plasma and erythrocytes). Next, 5 ml of 2:1 (v:v) chloroform to methanol containing 50 mg/l butylated hydroxytoluene (BHT) was added followed by 1 ml of 1 M NaCl. Samples were vortexed and centrifuged at 850 x g for 10 min at RT. The lower phase containing the total lipid extract was collected by aspiration and dried under nitrogen at 40°C.

2.12.2 Separation of the phosphatidylcholine (PC) lipid class from plasma samples by solid phase extraction (SPE)

The PC lipid class was only isolated from total lipid extracted from plasma samples, using the method described by Burdge et al., 2000 (267). For all other sample types the total lipid extract was used directly for the preparation of fatty acid methyl esters (FAMEs) described in section 2.12.3. Aminopropylsilica cartridges (Agilent, USA) were placed on an SPE tank connected to a vacuum pump and 2 ml of chloroform was added to each cartridge and allowed to drip through under gravity into a collection tube. The total lipid extract was dissolved in 1 ml of chloroform, added to the cartridge and allowed to drip through under gravity. After no more drips fell, the remaining liquid was drawn through the cartridge under vacuum. The cartridge was washed with 2 ml chloroform under vacuum and the solution collected was discarded. A new collection tube was placed in the SPE tank and 2 ml chloroform:methanol (60:40, v:v) was passed through the cartridges under vacuum, eluting the PC fraction, which was dried under nitrogen at 40°C.
2.12.3 Preparation of fatty acid methyl esters (FAMES)

Total lipid extracts and purified PC fractions were dissolved in 500 µl of toluene and vortex mixed. 1 ml of methylation reagent (methanol containing 2 % (v/v) H₂SO₄) was added and mixed gently. Tubes were heated at 50°C for 2 h. After cooling, 1 ml of neutralising solution (0.5 M KHCO₃ and 0.25 M K₂CO₃) was added followed by 1 ml of hexane. Samples were vortexed and centrifuged at 200 x g for 2 min at RT. The upper solvent phase containing the FAMES was removed and dried under nitrogen at 40°C. Each dried sample was reconstituted in 150 µl of hexane and transferred to a glass GC auto-sampler vial.

2.12.4 FAME analysis using gas chromatography (GC)

FAMES were separated using an Agilent J&W CP-Sil 88 capillary column (100 m length, 0.25 mm diameter and 0.2 µm film thickness) on an Agilent 6890 gas chromatograph (Agilent, UK) equipped with a flame ionisation detector (FID). The injector and detector temperatures were set at 255°C. The oven temperature was set to start at 70°C, ramped at 8°C/min to 110°C, ramped at 5°C/min to 170°C and held for 10 min, ramped at 4°C/min to 240°C and held for 16.5 min and then ramped at 60°C/min to 250°C and held for 10 min. Helium was used as the carrier gas. FAMES were analysed using Chemstation software (Agilent) and were identified by comparison of their retention times with that of standards containing known mixtures of FAMES: 37 FAMES, Menhaden oil and an n-3 preparation made by mixing 18:3n-3, 18:4n-3 and 20:3n-3 and an n-6 mix containing 22:4n-6 and 22:5n-6 (all purchased from Sigma-Aldrich). FAMES were quantified using the area of the internal standard.

2.12.5 Measuring stable isotope enrichment using GC-C-IRMS

FAMES were separated on a gas chromatograph (Thermo trace GC ultra, Bremen, Germany) equipped with an Agilent J&W DB-wax capillary column (30 m length, 0.25 mm diameter and 0.25 µm film thickness). The injector was set at 250°C. The initial oven temperature was set at 120°C for 1 min, then ramped at 25°C/min to 180°C and held for 32.5 min and then ramped at 25°C/min to 240°C and held for 13.7 min. Effluent of the GC was carried by helium carrier gas into a high temperature (940°C) combustion furnace where FAMES were oxidatively combusted to CO₂ molecules, which were then ionised and detected using a Thermo Finnigan Delta Plus XP IRMS. Faraday cups are positioned to simultaneously measure ion currents at mass to charge ratios (m/z) of 44, 45 and 46, representative of ¹²C₁⁶O₂, ¹³C₁⁶O₂ + ¹²C¹⁷O₁⁶O and ¹²C₁⁸O₁⁶O, respectively. The m/z 46 signal is used to correct the m/z 45 signal for the minor contribution of the ¹⁷O isotope, using a
correction algorithm based on the assumed relationship between $^{17}$O and $^{18}$O isotopic abundance, so as to recover the true $^{13}$C/$^{12}$C ratio.

The $^{13}$C/$^{12}$C ratios for identified fatty acids were measured relative to a laboratory reference gas standard calibrated to a reference FAME standard (Indiana University Standardised Methyl Ester) that had itself been standardised against the international standard Pee Dee Belemnite (PDB). Before the start of each run a zero enrichment test was performed in order to measure the reproducibility of the $^{13}$C/$^{12}$C ratio of the CO$_2$ reference gas by introducing ten pulses of reference gas and monitoring the standard deviation of the delta ($\delta$) values relative to a specified pulse; a mean standard deviation of <0.06 was accepted according to the manufacturer’s specifications. The background level of Argon (m/z 40) was also monitored before the start of each run to ensure there were no leaks in the system; a stable value of <100 mV was accepted. Peaks were identified by their retention times relative to the same standards described for GC-FID (section 2.12.4). The $\delta$ values of enriched ([$^{13}$C]18:3n-3 diluted 100 times with unlabelled 18:3n-3) and unenriched 18:3n-3 were also recorded for each run to monitor variability. Since PBMC numbers were limited, fatty acids extracted from erythrocytes (total lipid) and plasma (PC fraction) were converted to FAMEs and run on the GC-C-IRMS in order to obtain surrogate background $\delta$ values for each fatty acid being analysed.

2.12.5.1 GC-C-IRMS calculations

GC-C-IRMS data, expressed as delta ($\delta$) values were converted to $^{13}$C/$^{12}$C ratios using the equations detailed below (268). The standard $\delta$ notation describes the relative deviation of the $^{13}$C/$^{12}$C ratio in parts per thousand (‰) from PDB.

$$\delta^{13}\text{C}_{\text{PDB}} = \left[ \frac{R_{\text{SPL}} - R_{\text{PDB}}}{R_{\text{PDB}}} \right] \times 1000 = \left[ \frac{R_{\text{SPL}}}{R_{\text{PDB}}} - 1 \right] \times 1000$$

$R_{\text{SPL}}$ = $^{13}$C/$^{12}$C ratio in the sample

$R_{\text{PDB}}$ = $^{13}$C/$^{12}$C ratio in the PDB international standard (0.0112372)

$R_{\text{SPL}}$ is obtained by rearrangement of the equation: $R_{\text{SPL}} = (\delta^{13}\text{C}_{\text{PDB}}/1000 + 1) \times 0.0112372$

The percentage $^{13}$C abundance in each fatty acid is described using the atom percent (AP) and atom percent excess (APE). AP is calculated from the ratio using the following equation:

$$\text{AP} = 100 \times \left[ \frac{R_{\text{SPL}}}{R_{\text{SPL}} + 1} \right]$$
The AP corrected for background enrichment is called APE. The absolute amount of a specific labelled fatty acid is calculated by multiplying the APE by the total amount of that fatty acid (determined by GC-FID) per unit sample, which is most commonly mg of protein. A factor must be applied to correct for the presence of unlabelled carbons in the precursor and the addition of unlabelled carbons during fatty acid elongation and derivatisation to FAMES. For example, conversion of the tracer [1-13C]18:3n-3 to 20:5n-3 would result in one labelled carbon (from the precursor [1-13C]18:3n-3) out of the 21 total carbons (20 carbons in the fatty acid chain plus one carbon added during derivatization) being labelled with 13C. Therefore a correction factor of 21/1 would need to be applied to achieve the moles of labelled 20:5n-3. All data was normalised to the concentration of [1-13C]18:3n-3 in the PBMC growth medium (measured by GC-C-IRMS) to account for any slight differences that may have occurred when pipetting the stable isotope labelled 18:3n-3 into the PBMC growth medium.

2.12.5.2 GC-C-IRMS Standard Curve

A standard curve was prepared to test the GC-C-IRMS over a range of 13C/12C ratios. The standards were made using [U-13C]18:2n-6 due to the high cost and limited availability of [1-13C]18:3n-3. Individual stock solutions of [U-13C]18:2n-6 (16.68 µg/ml) and unlabelled 18:2n-6 (289.95 µg/ml) were prepared in chloroform. A series of defined amounts of [U-13C]18:2n-6 were pipetted into tubes followed by 300 µl (86.985 µg) of unlabelled 18:2n-6. The standards were dried under nitrogen at 40°C and methylated as described in section 2.12.4. The FAMEs were measured using GC-C-IRMS. The µmoles of [U-13C]18:2n-6 and unlabelled 18:2n-6 in each standard were calculated and multiplied by 18 to achieve the µmoles of 13C and 12C, respectively. The µmoles of 13C was divided by the µmoles of 12C and multiplied by a correction factor of 18/19 (to account for the carbon added to each fatty acid molecule during the methylation step) in order to calculate the expected 13C/12C ratio. The expected 13C/12C ratios were plotted against the 13C/12C ratios measured on the GC-C-IRMS (Figure 2.8). Perfect agreement would have a slope of 1x and for this standard curve a slope of 1.002x was achieved. The relationship was linear for delta values ranging from -28 to 2115.
2.12.5.3 Determination of dilution factor for labelled fatty acids with unlabelled fatty acids

A dilution experiment was carried out in order to find an appropriate ratio of labelled to unlabelled 18:2n-6 to add to cells for accurate measurement of ¹³C/¹²C ratios in cellular fatty acids using GC-C-IRMS. 1 mM stock solutions of [U-¹³C]18:2n-6 and unlabelled 18:2n-6 were prepared as described in Appendix A.2.2. [U-¹³C]18:2n-6 was then diluted with unlabelled 18:2n-6 at the following ratios: 1/10, 1/30, 1/60, 1/100. These dilutions were then added in triplicate to the media of Jurkat and HepG2 cell cultures to achieve a final concentration of 10 µM total 18:2n-6 (labelled and unlabelled) and incubated for 72 h. Additional replicates were treated with 10 µM of unlabelled 18:2n-6 as a background control.

Table 2.1 shows that the δ¹³C PDB (%) measurements for 18:2n-6, 20:3n-6, 20:4n-6 and 22:4n-6 increased as the dose of [U-¹³C]18:2n-6 was increased (except for the 1/100 dilution where δ¹³C PDB (%) for 18:2n-6 was slightly elevated in HepG2 cells relative to the 1/60 dilution). In HepG2 cells, the peak area for 22:4n-6 was below the detection limit.

GC-C-IRMS instruments are optimised to determine precise isotope ratios near natural abundance (δ¹³C equal to -35 to -5 ‰). As isotope ratios increase precision decreases but an arbitrary limit of δ¹³C less than 1500 ‰ can be set for measuring compounds enriched with stable isotopes without severely compromising precision (268, 269). For HepG2 cells all dilutions achieved delta values below 1500 ‰ for all labelled fatty acids measured (Table 2.1). In Jurkat cells this was only achieved using the 1/30, 1/60 and 1/100 dilutions, however, the 1/30 dilution was close to the detection limit for 18:2n-6. It was concluded from this experiment that [U-¹³C]18:2n-6 will need to be diluted.
by a factor of at least 1/60 with unlabelled 18:2n-6 before addition to cell culture experiments. The labelled 18:3n-3 used in future experiments has only one of the 18 carbons labelled with $^{13}$C and therefore it was calculated that the dilution factor should be approximately 18 times less (e.g. 1/3 instead of 1/60). However, when this was tested the delta values were higher than the arbitrary limit of 1500 ‰ and a dilution factor of 1/10 was sufficient for accurate measurement.

Table 2.1. Determination of optimal dilution of [U-$^{13}$C]18:2n-6 with unlabelled 18:2n-6 for precise measurement of delta values using GC-C-IRMS.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>HepG2</th>
<th>Jurkat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/10</td>
<td>1/30</td>
</tr>
<tr>
<td>$\delta^{13}$C PDB (‰)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>980.12</td>
<td>451.01</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>1395.36</td>
<td>759.49</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>633.99</td>
<td>240.83</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are the mean of three replicates from individual cell culture flasks. ND = not detectable.

2.13 Measurement of total cell protein

Total cell protein was extracted from a 20 µl aliquot of cells in 0.9 % NaCl, which were harvested as described in section 2.6.1. An equal volume of radioimmunoprecipitation assay (RIPA) buffer (Table 2.2) containing 10 µl/ml of Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) was added to the cell suspension and incubated on ice for 20 min. The cell suspension was then centrifuged at 16,000 x g for 10 min and the supernatant transferred to a clean 0.5 ml microcentrifuge tube. The protein concentration of samples was determined using a Pierce Protein Assay kit (Thermo Fisher Scientific) according manufacturer’s instructions for the microplate procedure. BSA standards were prepared at the following concentrations using RIPA buffer as the diluent: 2000, 1500, 1000, 750, 500, 250, 125, 25 and 0 (blank) µg/ml. 10 µl of each sample and standard were added in duplicate to a flat bottom 96 well plate and 200 µl of the working buffer provided with the kit was added. The plate was covered and incubated at 37°C for 30 min. Absorbance at 562 nm was measured using a spectrophotometer (thermolabsystems
A standard curve was prepared (Figure 2.9) by plotting the mean blank corrected 562 nm measurement for each BSA standard against its concentration in μg/ml. The standard curve was best fit with a quadratic equation as specified by the kit manufacturer. The concentration of protein in each sample was then determined using the standard curve.

Table 2.2. Composition of RIPA buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1 %</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Tris pH 8.0</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

![Typical BSA standard curve](image)

Figure 2.9. Typical BSA standard curve.
A BSA standard curve was used to determine protein concentration for samples measured using a Pierce Protein Assay kit. The curve was best fit with the quadratic equation $Y = -1 \times 10x^2 + 0.0007x + 0.005$. $R^2 = 0.9991$. 

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2.14 Nucleic acid extraction

2.14.1 Preparation of cultured cells for nucleic acid extraction

PBMC pellets were collected as described in section 2.6.1. For Jurkat cells the growth medium containing cells in suspension was collected and centrifuged at 300 x g for 5 min and the supernatant removed. The cell pellet was washed with 1 ml ice cold PBS and centrifuged again at 300 x g for 5 min. The PBS wash was repeated once more before the resulting cell pellet was stored at -80°C until required for RNA extraction.

2.14.2 RNA extraction

RNA was extracted from cell pellets using the Qiagen RNeasy® Mini kit (Qiagen) according to manufacturer’s instructions. An optional on column DNase digestion was carried out using the RNase-free DNase set (Qiagen) to eliminate genomic DNA contamination. RNA was eluted in 30 μl RNase-free water and its concentration was measured using a nanodrop1000 spectrophotometer (Labtech, UK) by measuring absorbance at 260 nm. RNA purity was assessed using the 260/280 and 260/230 ratios. The integrity of the RNA was checked using agarose gel electrophoresis described in section 2.14.4. The ribosomal RNAs (rRNA) 28S and 18S should appear as sharp bands (indicative of intact RNA). 28S rRNA and 18S rRNA are approximately 5 kb and 2 kb in size respectively. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. This integrity check relies on assumption that the rRNA quality reflects that of the mRNA which is in too low abundance to visualise. An example of extracted RNA (300 ng) analysed by agarose gel electrophoresis is given in Figure 2.10.

Figure 2.10. RNA quality inspected by agarose gel electrophoresis.
300 ng of RNA was analysed on a 1% agarose gel and ran at 120 volts. The 28S and 18S rRNA bands are indicative of intact RNA. Far left lane is 1 kb ladder followed by RNA from eight PBMC samples.
2.14.3 DNA extraction

DNA was extracted from cell pellets using the Qiagen DNeasy® blood and tissue kit according to the manufacturer’s instructions. The DNA was eluted in 30 μl RNase and DNase-free water and quantified using a nanodrop1000 spectrophotometer by measuring absorbance at 260 nm. DNA purity was assessed using the 260/280 and 260/230 ratios. DNA integrity was visually inspected by running 250 ng on an Agarose gel as described in section 2.14.4; degradation of DNA would result in a DNA ladder appearance. An example of extracted DNA (250 ng) analysed by agarose gel electrophoresis is given in Figure 2.11.

![DNA electrophoresis image](image)

Figure 2.11. DNA quality inspected by agarose gel electrophoresis.
250 ng of DNA was analysed on a 0.8% agarose gel and ran at 120 volts. Far left lane is 1 kb ladder followed by DNA from eight PBMC samples.

2.14.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to assess DNA and RNA quality and also the size and quality of PCR product. To make the gel, agarose (Sigma-Aldrich) was dissolved in 1 X Tris-acetate-EDTA (TAE) buffer (Fisher Scientific) by heating in a microwave. The following percentage (w/v) gels were used for running genomic DNA, RNA and PCR product, respectively: 0.8%, 1% and 2%. 7 μl of the nucleic acid stain GelRed (Cambridge Bioscience) was added prior to pouring the gel into a mould containing combs for casting wells and then the gel was left to set for approximately 15 min. The set gel was inserted into a gel tank containing TAE as a running buffer. Samples were mixed with BlueJuice™ (Thermo Fisher Scientific) gel loading buffer (10X) to achieve a 1X concentration and loaded into the gel. 5 μl of either a 1 kb or 100 bp ladder (Appendix A.5) was loaded depending on the size of the material being analysed. The gels were run at 120 volts and stopped when the loading dye had migrated a suitable distance for each application.
2.15 Real-time RT-PCR

The protocol detailed below for the analysis of relative gene expression by real-time RT-PCR was based on the method described in Hoile et al., 2014 (261).

2.15.1 Complementary DNA (cDNA) Synthesis

Complementary DNA (cDNA) was synthesised by reverse transcription. 1 μl of a dNTPs mix (Promega), containing equimolar amounts of each dNTP (10 mM) and 1 μl of random nonamers (Sigma-Aldrich) at 50 μM were added to 500 ng of RNA in a total volume of 10 μl and incubated for 10 min at 70°C. Samples were then placed on wet ice for at least 1 min. A master mix containing 4 μl 5 X M-MLV reaction buffer, 1 μl M-MLV Reverse Transcriptase (200 units/μl) and 5 μl RNase-free water was prepared and added to each sample (10 μl). Samples were transferred to a Veriti Thermal Cycler (Applied Biosciences, USA) where they were incubated at 15°C for 10 min, followed by 60 min at 37°C and then 10 min at 90°C to denature the reverse transcriptase. The newly synthesised cDNA was diluted with RNase free water to a total volume of 100 μl and stored at -20°C.

2.15.2 Real-time RT-PCR primers

Qiagen Quantitect primer assays, which contain a mix of forward and reverse primers for a specific gene were used to measure each gene of interest by real-time RT-PCR (Table 2.3). Upon arrival the Quantitect primers were reconstituted in 1.1 ml tris-EDTA (TE) buffer (Promega) to obtain a 10X assay solution. Qiagen does not disclose the primer sequences, however, bioinformatic information was available and all Quantitect primer assays used were designed to cross exon/exon boundaries and therefore should not amplify genomic DNA. Primers for the reference genes were obtained from Primer design again as ready-made mixes of forward and reverse primers. Reference genes are used to normalise data to constitutively expressed genes that are not affected by the experimental conditions. The reference genes were selected using geNorm analysis (see section 2.15.3.2).
Table 2.3. Human primer assays used for real-time RT-PCR.

<table>
<thead>
<tr>
<th>Primer target</th>
<th>Primer Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADS1</td>
<td>Qiagen Hs_FADS1_2_SG Quantitect Primer Assay (QT02322621)</td>
</tr>
<tr>
<td>FADS2</td>
<td>Qiagen Hs_FADS2_1_SG Quantitect Primer Assay (QT00077175)</td>
</tr>
<tr>
<td>ELOVL2</td>
<td>Qiagen Hs_ELOVL2_1_SG Quantitect Primer Assay (QT00059017)</td>
</tr>
<tr>
<td>ELOVL4</td>
<td>Qiagen Hs_ELOVL4_1_SG Quantitect Primer Assay (QT00017283)</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>Qiagen Hs_ELOVL5_1_SG Quantitect Primer Assay (QT00096334)</td>
</tr>
<tr>
<td>DNMT1</td>
<td>Qiagen Hs_DNMT1_1_SG Quantitect Primer Assay (QT00034335)</td>
</tr>
<tr>
<td>DNMT3a</td>
<td>Qiagen Hs_DNMT3A_1_SG Quantitect Primer Assay (QT00090832)</td>
</tr>
<tr>
<td>DNMT3b</td>
<td>Qiagen Hs_DNMT3B_1_SG Quantitect Primer Assay (QT00032067)</td>
</tr>
<tr>
<td>EIF4A2</td>
<td>Primerdesign reference gene assay (HK-SY-hu)</td>
</tr>
<tr>
<td>RPL13A</td>
<td>Primerdesign reference gene assay (HK-SY-hu)</td>
</tr>
<tr>
<td>SDHA</td>
<td>Primerdesign reference gene assay (HK-SY-hu)</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Primerdesign reference gene assay (HK-SY-hu)</td>
</tr>
</tbody>
</table>

2.15.3 Real-time RT-PCR

cDNA was diluted to a final concentration of 5 ng/µl in RNase and DNase free water, assuming 100% conversion of RNA to cDNA. A series of six cDNA standards were made by serial dilutions of cDNA prepared from HepG2 cells representing input RNA of 15 ng, 7.5 ng, 3 ng, 1.5 ng, 0.15 ng, 0.075 ng and 0.015 ng. A master-mix for each primer set was made containing 5 µl of Quantifast SYBR green (Qiagen), 0.5 µl of each primer set and 1.5 µl of RNase and DNase free water. This was added to 3 µl of sample and standard cDNA, in duplicate, in a white 384 well plate to give a final volume of 10 µl. Each plate included two no template controls (ntcs) where the sample cDNA was replaced with 3 µl of RNase and DNase free water. Standards were always measured for all primer sets on a plate. Plates were analysed using a LightCycler® 480 system (Roche Diagnostics, Switzerland) with the following cycling conditions: 95°C for 15 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s. A melt curve step followed comprising 95°C for 15 s, 60°C for 1 min and a gradual increase up to 95°C.
The melt curve analysis was performed to verify amplification of a single PCR product and absence of primer dimers on the basis that different size fragments of DNA differ in their melting points. The melting curve graphically displays the change in fluorescence when double stranded DNA dissociates into single stranded DNA when its melting point ($T_m$) is reached. This results in a sudden decrease in fluorescence due to the release of SYBR green. An example of a melt curve is given in Figure 2.12.

2.15.3.1 Quantification of mRNA expression levels

Expression levels of the target genes were quantified using the relative standard curve method (270) as described below.

Standard curves were constructed for both the target and reference genes. The relative amount of each target and reference gene for each sample was then determined by interpolating from the standard curve. The value for the target gene was divided by the geometric mean of the reference genes in order to normalise the data for differences in input. An example standard curve is given in Figure 2.12.

**Figure 2.12. Standard curve and melt curve for FADS2.**

(A) *FADS2* standard curve: slope = -3.409. $R^2 = 0.991$. (B) *FADS2* melting curve showing amplification of one specific product for all samples and no primer dimer. Samples are represented by red lines and ntc's are represented by blue lines.
**2.15.3.2 GeNorm analysis for selection of most suitable reference genes**

A GeNorm kit (Primerdesign) was used to determine the best candidate reference genes for normalisation in real-time RT-PCR experiments. Real-time RT-PCR was carried out for a panel of six reference genes (Figure 2.13) on cDNA from a representative set of samples (six replicates) for each comparative analysis of mRNA expression. The reference genes were ranked in order of expression stability using qbase+ (Biogazelle, Belgium) real-time RT-PCR data analysis software. The software generated two graphs, the first (Figure 2.13, A) shows the average expression stability value (geNorm M) for each reference gene, starting with least stable on the left and the most stable on the right. The second graph (Figure 2.13, B) was used to determine the optimum number of reference genes to include; the graph shows sequential inclusion of each reference gene, the optimum number being when the geNorm V value drops below 0.15. The optimal number of reference targets was given as four (geNorm V < 0.15). The three reference genes with the lowest geNorm M \((RPL13A, SDHA, EIF4A2)\) were selected. For the fourth reference gene 18S was selected instead of B2M since 18S rRNA level has been shown to remain unchanged in stimulated compared with unstimulated T-lymphocytes (271).
Figure 2.13. Graphs generated by geNorm analysis software (qbase+).
(A) Graph of average expression stability value (geNorm M) for each reference gene. (B) Graph showing the optimum number of reference genes; found when geNorm V is less than 0.15 (green line).

2.16 Pyrosequencing

Sodium bisulphite pyrosequencing was used to measure the DNA methylation status of 52 and 26 CpG dinucleotides in the regions adjacent to the human FADS2 and FADS1 transcription start sites (TSS), respectively, using the method described in Hoile et al., 2014 (261). The location of the FADS2 TSS was obtained from a publication (272) and the location of the FADS1 TSS was identified from the published sequence in the Ensembl genome browser (Ensembl transcript ID: ENST00000350997.11). The CpG dinucleotides measured were in a region between -18 and -1661 from the TSS for FADS2 (Figure 2.15) and +64 and -713 bp from the TSS for FADS1 (Figure 2.16). DNA methylation levels of nine CpG dinucleotides were also measured in a putative enhancer region (273) located in the intergenic region 7397 bp and 3202 bp from FADS2 and FADS1, respectively (Figure 2.17).
2.16.1 Sodium bisulphite conversion of DNA

Sodium bisulphite conversion of genomic DNA was used to differentiate unmethylated versus methylated cytosines. Bisulphite conversion of DNA (500 ng) was carried out using the Zymo Research EZ-96 DNA Methylation-Gold™ Kit (Cambridge Bioscience, UK) according to the manufacturer’s instructions. Bisulphite converted DNA was eluted in 30 μl RNase and DNase free water and stored at -20°C for subsequent PCR amplification.

2.16.2 PCR of bisulphite converted DNA

Prior to pyrosequencing, the genomic regions of interest were amplified using PCR.

2.16.2.1 Primer design and optimisation

Primer sequences were designed using PSQ Assay design software (Biotage, Sweden) and were ordered from Eurofins (Luxembourg), supplied at a stock concentration of 100 μM. Upon arrival, primers were stored at -20°C upon and were diluted to a working concentration of 10 μM prior to use. Primers sequences can be found in Table 2.4. The positions of the CpG dinucleotides measured are given relative to the reported transcription start sites of FADS2 and FADS1. For analysis of the intergenic region between FADS2 and FADS1, genomic coordinates (assembly GRCh38 from the Genome Reference Consortium) for the CpG dinucleotides are given. One of each primer pair was labelled with biotin at the 5’ end, depending on the assay design. This biotin labelled primer is incorporated into the PCR fragment while the amplification takes place and the biotinylated strand then serves as the template in the downstream pyrosequencing reaction. An annealing temperature gradient was run for each primer pair to determine which temperature gave the strongest single band of the correct size when the PCR products were analysed using gel electrophoresis.

2.16.2.2 PCR amplification of pyrosequencing template

PCR was carried out on bisulphite converted DNA using the primers listed in Table 2.4. For each PCR a reaction mix was set up containing 12.5 μl of 2 X Kapa2G Robust HotStart ReadyMix (Kapa Biosystems), 0.5 μl of each 10 μM forward and reverse primers (0.4 μM final concentration) and 10.5 μl of RNase and DNase free water. The reaction mix (24 μl) was added to 1 μl of bisulphite converted DNA in a 96 well plate giving a final volume of 25 μl. Two ntc’s were also prepared for each primer pair where the master mix was added to 1 μl of RNase and DNase free water instead of DNA. Cycling conditions were as follows: 95°C for 3 min (initial denaturation) then 45 cycles of 95°C for 15 s (denaturation), amplicon specific annealing temperature (Table 2.5) for 15 s, 72°C for 15 sec (extension) followed by a final extension step of 72°C for 1 min. The PCR products were run
on a 2% agarose gel to assess the size and quality of the amplified product (example given in Figure 2.14).

![Amplicon: FADS2/FADS1 Intergenic CpGs 61820621 to 61820755](image)

**Figure 2.14. Example agarose gel electrophoresis of pyrosequencing PCR product.**
The gel shows four PBMC samples followed by two ntc's and 1 kb DNA ladder.

### 2.16.1 Primers for pyrosequencing

All sequencing primers were designed using PSQ Assay Design software and were ordered from Eurofins, supplied at a concentration of 100 μM and stored at -20°C upon arrival. The primers were diluted to a 10 μM working concentration prior to use. Details of all sequencing primers are given in Table 2.5. Each sequencing primer was initially optimised in pyrosequencing reactions containing either 5 μl or 10 μl of PCR product and the volume chosen is given in Table 2.5. The FADS2 and FADS1 assays were based on previously published sequences (261).

#### 2.16.2 Immobilisation of PCR Product to Streptavidin Beads

The optimised volume of PCR product was added to a 96 well plate and made up to a volume of 40 μl with high purity water. A master mix containing 2 μl of streptavidin-coated sepharose beads (GE healthcare) and 38 μl of binding buffer (Qiagen), was prepared and 40 μl dispensed into each well. The plate was sealed and shaken on a plate shaker for 5 min at RT to allow capture of the biotinylated PCR product on streptavidin coated beads.
2.16.3 Preparation of single stranded pyrosequencing template DNA

For each sequencing primer a master mix containing 0.5 μl sequencing primer and 11.5 μl annealing buffer (Qiagen) was made and 12 μl was dispensed into the appropriate well of a PSQ HS 96 well plate (Qiagen). The plate was then inserted into the holding bay of the Pyromark Q96 Vacuum Prep Workstation (Biotage). Vacuum pressure was applied and the beads with immobilised PCR product were picked up from the 96 well plate by the vacuum prep tool. The vacuum prep tool with the attached beads was then washed by aspirating 70% ethanol through the filter probes. The vacuum prep tool was then immersed in denaturation solution (0.2 M NaOH) to separate the double stranded DNA into single strands. During this step the 5’ biotinylated strand remains immobilised on the beads while the released single strand is filtered out. The vacuum prep tool was next placed in wash solution (Qiagen) and the beads were washed by aspiration of the solution. The vacuum prep tool was then aligned with the PSQ plate before switching off the vacuum pressure and lowering into the PSQ plate. The beads with attached single stranded templates were released into the plate by gentle shaking.

2.16.4 Primer annealing and pyrosequencing

The PSQ plate was heated at 80°C for 2 min and allowed to cool for at least 5 min in order to allow the sequencing primers to anneal to the single stranded template DNA. For each pyrosequencing assay, the volume of nucleotides, enzyme and substrate (Qiagen PyroMark Gold Q96 reagents) required for each run was calculated by the software. The required amount was added to the appropriate PyroMark HS capillary dispensing tip (Qiagen) ensuring no bubbles were present. The tips were then inserted into dispensing tip holder, which was then placed in the pyrosequencer (Biotage PyroMark MD, Sweden). A tip test was carried out to ensure all tips were dispensing accurately. Finally, the PSQ plate was inserted into the pyrosequencer and the pyrosequencing run was started. Percent DNA methylation was analysed using Pyro Q CpG software. For each pyrosequencing assay a 0% and 100% methylated (Millipore) control DNA sample was processed alongside the samples and run each time. At least one bisulphite conversion control was included in each assay run file. This is a cytosine not followed by a guanine and should have been converted to a thymine (100% thymine) during bisulphite treatment and consequently can be used as a control for complete bisulphite conversion.
The underlined region is a CpG island.

**Figure 2.15. Location of CpG dinucleotides analysed by pyrosequencing relative to FADS2 TSS.**

The TSS (blue), translation start codon (orange) and CpG dinucleotides (yellow) are highlighted with measured CpG dinucleotides in bold font and their position (bp) relative to the FADS2 TSS is written above. The underlined region is a CpG island.

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Figure 2.16. Location of CpG dinucleotides analysed by pyrosequencing relative to FADS1 TSS. The TSS (blue), translation start codon (orange) and CpG dinucleotides (yellow) are highlighted with measured CpG dinucleotides in bold font and their position (bp) relative to the FADS1 TSS is written above. The underlined region is a CpG island.
Figure 2.17. Location of CpG dinucleotides in the FADS2/FADS1 intergenic region analysed by pyrosequencing.

Measured CpG dinucleotides are highlighted yellow, in bold font and with the chromosomal coordinate (assembly GRCh38) written above.
Table 2.4. Pyrosequencing PCR primers.

<table>
<thead>
<tr>
<th>CpGs Covered</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Amplicon Size (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FADS2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1661 to -1655</td>
<td>GTATGGTGTTTGGAGATTGTT</td>
<td>*AAAATACTCCCTAATTTCTACTTTCAACTA</td>
<td>168</td>
<td>54</td>
</tr>
<tr>
<td>-1337 to -1156</td>
<td>TTTTGTGAAATTTAGATTGAGG</td>
<td>*CCTAAAAAAATAACCTAACTACAT</td>
<td>290</td>
<td>54</td>
</tr>
<tr>
<td>-1119 to -1056</td>
<td>*ATTTGAGGTTTTTATAATTTTTTATGAT</td>
<td>ACCCTAAATCTCAATAAACCTCAACTCT</td>
<td>319</td>
<td>56</td>
</tr>
<tr>
<td>-1013 to -975</td>
<td>GGGTAGTGTGTTGTTGTTGAGTT</td>
<td>*TACACCCCCACCTCTCTAT</td>
<td>221</td>
<td>58</td>
</tr>
<tr>
<td>-914 to -855</td>
<td>GGTAGTTTTATATTGGAGGTTGAT</td>
<td>*AAACCTCTACTCTACTTTTCTTAATCT</td>
<td>199</td>
<td>56</td>
</tr>
<tr>
<td>-817 to -775</td>
<td>*ATTGAGTTATGAGATTTAGGGTAAGG</td>
<td>ACTTTAACCCCTCTCAAAACAATCTT</td>
<td>167</td>
<td>56</td>
</tr>
<tr>
<td>-718 to -667</td>
<td>ATTTGTTGGATTAGGTTTTTAAAGTT</td>
<td>*AAACTCCCAAATACCCACATT</td>
<td>116</td>
<td>54</td>
</tr>
<tr>
<td>-374 to -334</td>
<td>GGATAATGTTGGATTTGGAAGTT</td>
<td>*CCTACCATTAAACCCAAAAAAATCTTCC</td>
<td>408</td>
<td>60</td>
</tr>
<tr>
<td>-258 to -84</td>
<td>AAGATTTTTTTGGTTAATGTT</td>
<td>*AAATCCCTAACTTCCTCCTAAAC</td>
<td>263</td>
<td>56</td>
</tr>
<tr>
<td>-230 to -133</td>
<td>GAAGATGTTTTTTGGTTAATGTTAG</td>
<td>*ATCCCTAATCTCCCAATACC</td>
<td>263</td>
<td>60</td>
</tr>
<tr>
<td>-64 to -50</td>
<td>*GGGGAGTTTTTATTGGAGGTAAGG</td>
<td>AATCCCTAATCTCCCAATACC</td>
<td>95</td>
<td>52</td>
</tr>
<tr>
<td>-18</td>
<td>TGGGGGTATTTGGAAGGTTAG</td>
<td>*CCTCCCCCAACCTTCTC</td>
<td>80</td>
<td>58</td>
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<tr>
<td><strong>FADS1</strong></td>
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<td>-713</td>
<td>TAGGGTTGTTAGGAGAATTAAATGAG</td>
<td>*AAACACTCTATTATACCAAAAAAACATT</td>
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<td>-581</td>
<td>AAGAATAGTGTTGGATATTAGAAATG</td>
<td>*TCTCCTAACCTTTAAATTTTACCT</td>
<td>279</td>
<td>50</td>
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<tr>
<td>-506</td>
<td>*AAGAATAGTGTTGGATATTAGAAATG</td>
<td>AACTCTCCTAACCTTTAAATTTTACCT</td>
<td>283</td>
<td>52</td>
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<tr>
<td>-430</td>
<td>AGGGTTTTTATAGGATTATAGTTAGGAA</td>
<td>*AAATTTTTATAACCTTTACCTTCTT</td>
<td>180</td>
<td>52</td>
</tr>
<tr>
<td>-262 to -188</td>
<td>AGAGGTATAGGTTATAGGTTAATAGTTAGT</td>
<td>*CTCTCAAAAAAATACCTACTACCTCTTA</td>
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<td>52</td>
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<td>-94 to -21</td>
<td>AGTGAATGGATATGGAGGTTAGG</td>
<td>*ACCCCAACCCACATCCACCA</td>
<td>139</td>
<td>58</td>
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<td>+17 to +64</td>
<td>GGAGGGTATAGGTTATTGGAAGG</td>
<td>*CCCCCATAAATCTAACAACCTCACAA</td>
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<td>52</td>
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<td><strong>FADS2/FADS1 intergenic region</strong></td>
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<tr>
<td>61820364 to 61820508</td>
<td>ATGGGTGTTAGGTTATAGTGTATTAGT</td>
<td>*AAACAAAAATCCCAACCCCTATCC</td>
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<td>60</td>
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<tr>
<td>61820621 to 61820755</td>
<td>GTTTGAGTTTTGTTAGGTTTAGGGAATTTTATA</td>
<td>*AACCTCACCCCCCAAAATAAC</td>
<td>232</td>
<td>60</td>
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<tr>
<td>61820814 to 61820848</td>
<td>GTTGGAGTTTTTATAGGTTATGTTA</td>
<td>*CACCCAAACTAAAATAACTAATAAAC</td>
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<td>60</td>
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The location of the biotin tag is indicated by *. 

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Table 2.5. Pyrosequencing sequencing primers.

<table>
<thead>
<tr>
<th>CpGs Measured</th>
<th>Sequencing primer (5’-3’)</th>
<th>Volume of PCR product added (µl)</th>
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<tr>
<td>-1337</td>
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<td>10</td>
</tr>
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<td>-1278</td>
<td>GGTGTTTTTTAATTTGAGGATG</td>
<td>10</td>
</tr>
<tr>
<td>-1156</td>
<td>GGGTTTTATATAAAAAATTAGT</td>
<td>10</td>
</tr>
<tr>
<td>-1119, -1112, 1101</td>
<td>AAAATAAACCTAATCAATCC</td>
<td>10</td>
</tr>
<tr>
<td>-1071, -1067, 1056</td>
<td>CCTCAACCCCAACT</td>
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<td>-1013</td>
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<td>-980, -975</td>
<td>GTAGATTTTTAGAAATAGGTTGCT</td>
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</tr>
<tr>
<td>-914</td>
<td>ATATTGTTTTATGAGGTTT</td>
<td>5</td>
</tr>
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</tr>
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</tr>
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<td>-775</td>
<td>CCTCTAATCAAACACTTAAAAA</td>
<td>5</td>
</tr>
<tr>
<td>-718</td>
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<td>-686</td>
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<td>10</td>
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<td>GGTAGGGTGTAGG</td>
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<td>-172, -167, -157, -149</td>
<td>GAAAGATTTTTTTTGGTTAATGAG</td>
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<td>-84</td>
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</tr>
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<td>-64, -50</td>
<td>CCCATATCCCCCAA</td>
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</tr>
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<td>-18</td>
<td>GGGGAGGTTGATTCT</td>
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<tr>
<td><strong>FADS1</strong></td>
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<td></td>
</tr>
<tr>
<td>-713</td>
<td>GGAGAATTTAATGAGGTTAAGG</td>
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<td>-581</td>
<td>AGTTTGGAATGGTTAATTTTA</td>
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<td>-506</td>
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<td>-430</td>
<td>AATATTAGGTTAGGAGG</td>
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<td>AGTTTTTTTTTTATTATTGAGT</td>
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<td>-188, -183, -180</td>
<td>TTGTATTTTAGGTTAGTATAATTG</td>
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<td>GGTAGGGGTGAGGTTT</td>
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<td>-51, -45, -30, -21</td>
<td>GATTTAGGAGGTAGGAG</td>
<td>5</td>
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<td>+17, +15, +23</td>
<td>AGTTTTGGTTTTTTTTTTATTAT</td>
<td>10</td>
</tr>
<tr>
<td>+47, +59, +61, +64</td>
<td>ATTTAGGTTTTGTAGGATTAA</td>
<td>10</td>
</tr>
<tr>
<td><strong>FADS2/FADS1 intergenic region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61820364</td>
<td>GGAGAGGATGTTAATGAGG</td>
<td>10</td>
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<tr>
<td>61820508</td>
<td>GGTGATGTTAGTTAGTTTTTAT</td>
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<td>61820621, 61820625</td>
<td>ATATAATTTGTTAGGTTAT</td>
<td>10</td>
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<td>61820674</td>
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<td>61820717</td>
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</tr>
<tr>
<td>61820755</td>
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<td>61820814</td>
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<td>10</td>
</tr>
<tr>
<td>61820848</td>
<td>AAAGGTTTAGGAGT</td>
<td>10</td>
</tr>
</tbody>
</table>
2.17 5’ Rapid Amplification of cDNA Ends (5’ RACE)

5’ RACE enables amplification of nucleic acid sequences at the 5’-end of mRNA templates and therefore permits identification of the TSS. Duplicate PBMC cultures were prepared from purchased PBMCs; one stimulated with 5 μg/ml Con A the other without as described in section 2.6. 5’ RACE was also performed for Jurkat cells cultured as described in section 2.11.2. Cultures were harvested after 48 h and RNA was extracted as detailed in section 2.14.2. Two different 5’ RACE procedures were used: Ambion 5’ RNA ligase mediated RACE (5’ RLM RACE) and SMARTer® 5’ RACE (Clontech Laboratories).

2.17.1 5’ RLM RACE

5’ RLM RACE was carried out for 10 μg of input RNA according to manufacturer’s instructions. An overview of this process is given in Figure 2.18.

![Diagram of RLM 5’ RACE procedure](image)

**Figure 2.18. Overview of RLM 5’ RACE procedure.**
Calf intestine alkaline phosphatase (CIP); Tobacco acid pyrophosphatase (TAP). Adapted from the Ambion 5’ RLM RACE handbook.

2.17.1.1 Nested PCR for 5’ RLM RACE

The 5’ end of the FADS2 transcript was amplified by nested PCR. Two nested primer sequences corresponding to the 5’ RACE adapter sequence were provided with the 5’ RLM RACE kit. Two nested antisense primers (Gene Specific Inner and Outer primers) specific to the FADS2 transcript
were designed using Primer-BLAST software (NCBI, USA) ensuring they were as close to the 5’ end of the known transcript as possible in order to avoid an overly large amplification product. In addition, a forward FADS2 specific primer (Gene Specific 5’ Control primer) was designed to verify presence of the target. The positions of the gene specific primers are given in Figure 2.20. All primers (Table 2.6) were supplied by Eurofins at a concentration of 100 µM and were stored at -20°C upon arrival. 1 µl of 5’ RLM RACE cDNA was amplified in an outer PCR using 1.25 U Go Taq Hot Start DNA polymerase (Promega), 10 µl 5X Go Taq Flexi Buffer, 1.5 mM MgCl₂, forward and reverse primers at a final concentration of 0.4 µM and dNTPs at a final concentration of 200 µM each in a total volume of 50 µl with RNase and DNase free water. An annealing temperature gradient of 55°C, 60°C and 65°C was run for each primer set. PCR was carried out using a Veriti Thermal Cycler with the following cycling conditions: 94°C for 2 min (initial denaturation), followed by 35 cycles of 94°C for 30 s (denaturation), 60°C for 30 s (annealing) and 72°C for 1 min (extension) followed by a final extension at 72°C for 5 min. 1 µl of the outer PCR was used as a template for the inner PCR with the same reaction components and conditions.

### 2.17.2 SMARTer® 5’ RACE

SMARTer® 5’ RACE was carried out for 1 µg of input RNA according to manufacturer’s instructions. An overview of this process is given in Figure 2.19.

**Figure 2.19. Overview of SMARTer® 5’ RACE procedure.**
Reverse transcriptase (RT). Adapted from the Clontech Laboratories SMARTer® RACE 5’/3’ Kit User Manual.
2.17.2.1 SMARTer® 5’ RACE PCR

A universal primer mix designed to bind to the incorporated SMARTer II A Oligonucleotide sequence was provided with the SMARTer® 5’ RACE kit. An antisense primer (Table 2.6) specific to the FADS2 transcript (Gene Specific primer) was designed using Primer-BLAST software. The gene specific primer was designed to have a \( T_m > 70^\circ C \) to enable the use of touchdown PCR. The following sequence was added to the 5’–end of the gene specific primer for downstream In-Fusion cloning: GATTACGCCAAGCTT. SMARTer® 5’ RACE does not generally require nested PCR and therefore only one gene specific primer was designed. Presence of the target was verified using the same forward FADS2 specific primer (Gene Specific 5’ Control Primer) and Inner Gene Specific Primer designed for 5’ RLM RACE (section 2.17.1). 2.5 µl of RACE-ready cDNA was amplified using 1.25 U SeqAmp DNA polymerase, 25 µl 2X SeqAmp buffer, 5 µl 10X UPM, 1 µl Gene Specific Primer (final concentration of 0.2 µM) and 15.5 µl of nuclease free water to give a total volume of 50 µl. PCR was carried out using a Veriti Thermal Cycler with the cycling conditions below.

- 5 cycles:
  - 94°C 30 s
  - 72°C 3 min
- 5 cycles:
  - 94°C 30 s
  - 70°C 30 s
  - 72°C 3 min
- 25 cycles:
  - 94°C 30 s
  - 68°C 30 s
  - 72°C 3 min

2.17.3 Purification of amplified RACE products by gel extraction

PCR products were analysed using agarose gel electrophoresis and extracted from the gel using using a Zymoclean gel DNA recovery kit (Zymo Research), according to manufacturer’s instructions. Gel purified products were quantified using a nanodrop spectrophotometer and 300 ng was analysed using agarose gel electrophoresis to check purity.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene specific 5’ Control</td>
<td>ACAAAAGCGAAAGCGAAGAG</td>
</tr>
<tr>
<td>5’ RLM RACE Gene Specific Outer</td>
<td>TTGCATCTTCTCCAGGCTAGT</td>
</tr>
<tr>
<td>5’ RLM RACE Gene Specific Inner</td>
<td>GGTGCAGGTTATGCTTT</td>
</tr>
<tr>
<td>SMARTer® 5’ RACE Gene Specific</td>
<td>GATTACGCCAAGCTTGGGCTGCCATTCGCCAGAACAAACACG</td>
</tr>
</tbody>
</table>

The 15 bp sequence required for In-Fusion cloning is shown in bold.
Figure 2.20. Locations of FADS2 5’ RLM RACE primers. 
(A) Schematic of primer positions for 5’ RLM RACE showing size of control amplicons. (B) Schematic of primer positions for SMARTer® 5’ RACE. Note the gene specific inner primer from 5’ RLM RACE is used as the control primer to verify presence of FADS2 transcript. (C) Exons 1 to 5 (alternating black and red) of FADS2 mRNA transcript (Ensembl transcript ID: ENST00000278840.8). Only part of the 5’UTR (underlined) in exon 1 is shown. The positions of the forward 5’ gene specific control primer (green), reverse 5’ RLM RACE gene specific inner and outer primers in exon 1 and reverse SMARTer® 5’ RACE gene specific primer in exon 5 (all yellow) and the published TSS for FADS2 (blue) are highlighted.
2.17.4 Cloning 5’ RACE products for DNA sequencing

2.17.4.1 Bacterial cell culture media

Antibiotics:

An ampicillin stock solution was made at a concentration of 100 mg/ml, filter sterilised by passing through a 0.2 µm filter and then aliquoted into 1.5 ml tubes and stored at -20°C.

LB media:

20 g Luria broth (LB) powder was mixed with one litre of water and autoclaved (3 bar, 121°C, 15 min) to sterilise.

LB Agar plates:

20 g LB powder plus 15 g agar powder per litre of water were autoclaved to sterilise. The solution was allowed to cool to approximately 30°C before supplementing with 100 µg/ml ampicillin. 25 ml was poured into 90 mm petri dishes and allowed to cool. Plates were sealed and stored inverted (to prevent condensation forming on the agar) at 4°C until use.

2.17.4.2 Insertion of 5’ RLM RACE products into plasmid vector using TA Cloning®

5’ RLM RACE PCR products were cloned using the TOPO® TA Cloning® Kit for Sequencing (Thermo Fisher). The plasmid vector pCR™4-TOPO® (Figure 2.21) provided with the kit is supplied linearised with single overhanging 3’ deoxythymidine (T) residues. PCR products produced by Taq DNA polymerase have a single deoxyadenosine (A) added to the 3’ ends due to its terminal transferase activity. This allows PCR products to ligate efficiently with the pCR™4-TOPO® plasmid vector by a method called TA Cloning®. The cloning reaction was carried out using 1 µl PCR product, 1 µl salt solution (provided with kit), 3 µl nuclease free water and 1 µl pCR™4-TOPO® plasmid vector giving a final volume of 6 µl. The reaction components were mixed gently and incubated for 5 min at RT and then placed on ice.

2.17.4.3 Transformation of pCR™4-TOPO® construct into chemically competent E. coli

After performing the cloning reaction the pCR™4-TOPO® construct was transformed into One Shot® TOP10 chemically competent E. coli. 2 µl of the TOPO cloning reaction was added to one vial of One Shot® TOP10 chemically competent E. coli, mixed gently and then incubated on ice for 5 min. The cells were heat shocked at 42°C for 30 s then transferred to ice immediately. 250 µl of RT SOC medium (provided with kit) was added and cultures were shaken horizontally at 37°C, 200 rpm for
1 h. 50 µl of suspended cells from each transformation was spread onto a pre-warmed ampicillin agar plate and incubated at 37ºC overnight. The plasmid vector contains the ampicillin resistance gene allowing transformants to be distinguished from non-transformants by their ability to survive on the ampicillin containing agar. Four single colonies were picked and grown in 5 ml LB media supplemented with 100 µg/ml ampicillin in a shaking incubator at 37ºC overnight.

2.17.4.4 Insertion of SMARTer 5’ RACE products into plasmid vector using In-Fusion Cloning

SMARTer® 5’ RACE products were cloned using the In-Fusion cloning kit, which was provided as part of the SMARTer® 5’/3’ RACE kit. In-Fusion cloning is a ligation-independent cloning method based on the annealing of complementary ends of a cloning insert and linearised cloning vector. The plasmid vector pRACE (Figure 2.22) provided with the kit is supplied linearised and contains 15 bp extensions complementary to the PCR product ends. Single stranded 15 nucleotide overhangs at the termini of the cloning insert and linearised cloning vector are generated by the In-Fusion enzyme mix and these overhangs are annealed at the sites of complementarity. The cloning reaction was carried out using 1 µl (50 ng) pRACE vector, 7 µl (150 ng) gel-purified RACE product and 2 µl In-Fusion HD Master Mix. The reaction components were mixed gently and incubated for 15 min at 50 ºC and then placed on ice.

2.17.4.5 Transformation of pRACE construct into Stellar Competent Cells

After performing the cloning reaction the pRACE construct was transformed into Stellar Competent Cells. 2.5 µl of the In-Fusion cloning reaction was added to 50 µl of Stellar Competent Cells, mixed gently and then incubated on ice for 30 min. The cells were heat shocked at 42ºC for 45 s then transferred to ice immediately. Pre-warmed SOC medium (37 ºC) was added to bring the final volume to 500 µl and cultures were shaken horizontally at 37ºC, 200 rpm for 1 h. 50 µl of each transformation reaction was spread onto a pre-warmed ampicillin agar plate and incubated at 37ºC overnight. Eleven single colonies were picked and grown in 5 ml LB media supplemented with 100 µg/ml ampicillin in a shaking incubator (200 rpm) at 37ºC overnight.

2.17.4.6 Plasmid preparations and restriction enzyme digest

1.5 ml of bacterial cultures from sections 2.17.4.3 and 2.17.4.5 were pelleted by centrifugation at 7000 x g for 3 min. Plasmid DNA was then purified using a QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer’s instructions. Plasmid DNA was eluted in 50 µl RNase and DNase free water and quantity and purity was measured using a NanoDrop spectrophotometer. To confirm presence of the 5’ RACE PCR product insert in the plasmid vector a restriction endonuclease digest was performed. 500 ng of the pCR™4-TOPO® construct was digested with 1 µl of EcoRI, and 300 ng
of the pRACE construct was digested with 0.5 µl EcoRI and 0.5 µl HindIII (FastDigest Restriction Enzymes, Thermo Fisher Scientific). Digests were performed in 1 µl 10 X FastDigest buffer and RNase and DNase free water (to achieve a total reaction volume of 10 µl) and incubated at 37 °C for 30 min. The digested plasmid DNA was analysed by agarose gel electrophoresis to check the appropriate sized insert had been cut out of the digested vector.

### 2.17.4.7 Sequencing RACE products

Between 600 ng and 2000 ng of each 5’ RACE clone was sent to GATC Biotech (Germany) for sequencing. The T3 and T7 sequencing primers (Figure 2.21) were selected for forward and reverse sequencing reads of pCR™4-TOPO® constructs. The M13 forward and M13 reverse sequencing primers were selected for sequencing of pRACE constructs (Figure 2.22).

![Figure 2.21. Map of pCR™4-TOPO® plasmid and sequence surrounding the TOPO® cloning site.](image)

**Figure 2.21.** Map of pCR™4-TOPO® plasmid and sequence surrounding the TOPO® cloning site. (A) Circular map showing the features of the pCR™4-TOPO® vector including the ampicillin resistance coding region used for selection of transformants and the TOPO® cloning site. (B) Detailed view of the sequence surrounding the TOPO® cloning site including 3’ T overhangs, T3 and T7 priming sites and EcoRI restriction sites. Taken from the TOPO® TA Cloning® Kit for Sequencing product manual (Thermo Fisher Scientific).
Figure 2.22. pRACE vector map and In-Fusion cloning site.
Circular map showing the features of the pRACE vector including the ampicillin resistance coding region used for selection of transformants and the In-Fusion cloning site. A view of the sequence surrounding the In-Fusion cloning site is given below showing the EcoRI and HindIII restriction sites and the regions of homology to the universal primer mix (UPM) and gene specific primer (GSP). Taken from the pRACE vector map document produced by Clontech Laboratories.
2.18 Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics 22.0 (IBM, USA) and GraphPad Prism 6 (GraphPad software, USA). All data are expressed as mean ± standard error of the mean (SEM) or median ± interquartile range (IQR). Statistical significance was set at P < 0.05. Normality of data was assessed both numerically using Kolmogorov-Smirnov and Schapiro-Wilk tests and graphically by inspecting histograms and normal Q-Q plots. Data that were not normally distributed were analysed with the appropriate non-parametric statistical test. Details of individual statistical tests are given in Figure legends. Comparisons between unstimulated and Con A stimulated PBMCs were analysed as paired data as the PBMCs were derived from the same individual and cultured in media supplemented with autologous plasma. Paired data are displayed in graphs with connecting lines and also in graphs displaying difference scores.
Chapter 3: Does activation of PBMCs increase the activity of the PUFA biosynthesis pathway?
3.1 Introduction

In mammals, conversion of 18:3n-3 to longer chain n-3 PUFAs involves the sequential action of desaturase (delta-6 desaturase and delta-5 desaturase) and fatty acid elongase (elongase-5 and elongase-2) enzymes followed by one cycle of fatty acid β-oxidation to form 22:6n-3 (the details of this pathway are given in section 1.4.1 and Figure 1.1). The conventional pathway begins with delta-6 desaturation of 18:3n-3 to yield 18:4n-3. However, as detailed in section 1.4.4 and Figure 1.2, an alternative pathway, consisting of elongation of 18:3n-3 to yield 20:3n-3 followed by desaturation at the delta-8 position to form 20:4n-3 (a product in the conventional pathway), has been reported (19, 33).

Activation of T-lymphocytes is a complex process involving marked changes to the morphology, biochemistry and function of the cells resulting in mature blast cells that can divide to generate effector T-lymphocytes (section 1.9). In this process T-lymphocytes switch their metabolic programme from β-oxidation and catabolic metabolism to aerobic glycolysis and anabolic metabolism (274). As discussed in section 1.9.2, T-lymphocyte proliferation can be activated in vitro by a number of different agents including non-specific mitogens such as the plant lectins Con A and PHA. Con A binds to α-mannose and α-glucose containing glycoprotein or glycolipid receptors on the cell surface, inducing activation of T-lymphocytes and acts as an in vitro model for activation of lymphocytes by specific antigens (152).

T-lymphocyte activation involves rapid changes in the phospholipid metabolism of plasma membranes including de novo synthesis and changes in the fatty acid moieties of membrane phospholipids (137-139) and phospholipid hydrolysis to generate lipid second messengers (134). Exogenous fatty acids are essential for in vitro activation and continued proliferation of T-lymphocytes (144, 145). In vitro experiments have demonstrated that fatty acid uptake is increased in activated T-lymphocytes and this has been shown to include the n-3 PUFAs 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3, when available in the culture medium (40, 49). However, at high doses the fatty acids, particularly n-3 PUFAs, begin to exert inhibitory effects on lymphocyte proliferation in vitro (163, 164, 275).

A recent study in CD4+ T-lymphocytes demonstrated that both fatty acid uptake and de novo biosynthesis pathways are required for robust proliferation after antigenic stimulation; whereas inhibition of either results in insufficient proliferation and inhibition of both leads to apoptosis (146). As discussed in section 1.10, n-3 PUFAs have important effects on lymphocyte function (135) but the ability of mitogen stimulated lymphocytes to synthesise these fatty acids has only been investigated in a single study (147). The researchers measured n-3 PUFA synthesis in PBMCs from healthy individuals using the radiolabelled precursor [1-14C]18:3n-3. Treatment of PBMCs with the
T-lymphocyte mitogen PHA progressively increased delta-6 and delta-5 desaturase activity (147). Radioactivity recovered in tetraene fraction (18:4n-3 and 20:4n-3 combined) after 72 h was low for both unstimulated and PHA stimulated cells, at 1.5% and 1.4%, respectively. Activity of both delta-5 and delta-6 desaturases increased in activated cells, estimated from the radioactivity recovered in the pentaene fraction (20:5n-3 and 22:5n-3 combined), which was 5.4% for non-activated and 14.4% for activated cells. No radioactivity was observed in the hexaene fraction indicating no synthesis of 22:6n-3. The study also showed that supplementation with 30 µM unlabelled 18:3n-3 significantly raised the proportion of its elongation product 20:3n-3 in activated cells and the n-6 PUFA pathway equivalent to 20:3n-3 (20:2n-6) also increased after supplementation with 30 µM unlabelled 18:2n-6. There were no significant changes in the proportions of other n-3 PUFA derived from 18:3n-3 but their total amount increased, indicating the existence of desaturase activities in activated cells (147). The authors of this study concluded that the importance of n-3 PUFA synthesis relative to fatty acid uptake in the lipid compositional changes that occur in activated lymphocytes is small, merely representing a fine-tuning of the final fatty acid composition of cell phospholipids for synthesis of new cell membranes. However, this study was limited by a small sample number and did not report findings for individual radiolabeled fatty acids, only combined fractions.

Aims

The aims of this chapter are to determine whether the previous observation of increased n-3 PUFA synthesis in activated human PBMCs is repeatable and whether inhibition of n-3 PUFA synthesis affects proliferation.

Hypotheses

- CD69 expression in PBMCs is higher in response to activation.
- Synthesis of individual n-3 PUFAs in PBMCs is higher in response to activation.
- Inhibition of the n-3/n-6 PUFA biosynthesis pathway decreases the proliferation of lymphocytes.

To address these hypotheses, the expression of the activation marker CD69 and the levels of individual n-3 PUFAs synthesised from stable isotope labelled 18:3n-3 were measured in PBMCs cultured in the presence and absence of Con A. For clarity, figures show combined male and female data. The results of statistical analysis of individual male and female groups are given in the text. Since measurement of n-3 PUFA synthesis required addition of 18:3n-3 to the culture medium, the effect of 18:3n-3 supplementation on PBMC proliferation and viability was examined first. Finally, Con A stimulated PBMCs were treated with the delta-6 desaturase inhibitor SC-26196 to determine how pathway inhibition affects proliferation.
3.2 Methods

3.2.1 Sample collection, PBMC Isolation and measurement of cell specific surface markers

Volunteers were recruited as part of the EPUFA study as described in section 2.2. Anthropometric measurements were taken (section 2.2.5) and plasma TG, total cholesterol, HDL cholesterol and glucose concentration and blood total haemoglobin concentration were measured as detailed in section 2.3. PBMCs were isolated from lithium heparin blood (section 2.4) and the proportions of T-lymphocytes, B-lymphocytes and monocytes within the PBMC population were determined by measuring the expression of the cell surface markers CD3, CD19 and CD14, respectively, using flow cytometry as described in section 2.5. These markers were also analysed following cell culture.

3.2.2 PBMC culture and measurement of CD69 expression

PBMCs cultures treated with 20 µM stable isotope labelled 18:3n-3 ([1-13C]18:3n-3) diluted 1/10 with unlabelled 18:3n-3 and either no Con A or 5 µg/ml Con A, were maintained for 48 h as detailed in Figure 3.1. PBMCs were harvested as described in section 2.6.1. Activation was confirmed by measurement of CD69 positive events by flow cytometry (section 2.7).

![Figure 3.1. Experimental design for comparison of Con A stimulated and unstimulated PBMCs.](image)

3.2.3 Measurement of n-3 PUFA synthesis

PBMCs were harvested as described in section 2.6.1 and erythrocytes and plasma collected as described in section 2.4. Total lipid was extracted (section 2.12.1) and the PC lipid class was purified
from plasma total lipid extract using SPE (section 2.12.2). \(^{13}\text{C}\)-label incorporation into specific n-3 PUFAs in PBMCs was measured by a combination of GC and GC-C-IRMS as detailed in section 2.12.5 with normalisation to total cell protein (section 2.13). The average \(^{13}\text{C}/^{12}\text{C}\) ratio of erythrocyte total lipid and plasma PC fatty acids was utilised for background correction. Zero values represent either where the \(^{13}\text{C}/^{12}\text{C}\) ratio was the same as background or where no fatty acid peak was detected.

### 3.2.4 Viability and proliferation measurements

These experiments were carried out on purchased PBMCs that were revived and cultured as described in section 2.8.

To determine the effect of 18:3n-3 supplementation on PBMC viability, cultures were prepared either with or without 5 µg/ml Con A treatment and six replicate cultures of each were treated with 10 µM, 20 µM or 50 µM 18:3n-3 or 0.02 % (v/v) ethanol vehicle control for 48 h. Viability was measured using the LIVE/DEAD Red Dead Cell Stain Kit and flow cytometry as described in section 2.9.2. The same experimental design was used to measure the number of viable cells using the CellTiter-Glo Luminescent Cell Viability Assay as described in section 2.9.1. Proliferation analysis of PBMCs treated with 0 µM or 20 µM 18:3n-3 for 120 h, in the presence of 5 µg/ml Con A, was carried out using CFSE staining and flow cytometry as described in section 2.10.

The viability of Con A treated (5 µg/ml Con A) PBMCs, after 144 h of exposure to 100 nM and 200 nM SC-26196 compared to a 0.02 % (v/v) DMSO vehicle control was measured using the LIVE/DEAD Red Dead Cell Stain Kit and flow cytometry (section 2.9.2), with six replicate cultures for each treatment. Proliferation analysis of Con A treated (5 µg/ml) PBMCs incubated with 0 nM (0.02 % (v/v) DMSO vehicle control), 100 nM and 200 nM SC-26196 for 48 h, 72 h, 96 h, 120 h and 144 h was carried out using CFSE staining and flow cytometry as described in section 2.10. Six replicate cultures were performed for each SC-26196 concentration and time point. Cultures were also prepared without Con A treatment as a non-proliferating control.
3.3 Results

3.3.1 Study population characteristics

Table 3.1 shows the characteristics of males and females in the volunteer population used for comparison of n-3 PUFA synthesis in unstimulated and Con A stimulated PBMC cultures; this population was also used for comparisons of gene expression and DNA methylation in stimulated and unstimulated PBMCs, given in later chapters. A smaller population of volunteers was used for these comparisons since this aspect of the analysis required duplicate cultures, which was not feasible for the total EPUFA study population. Furthermore, a more homogeneous age range was chosen as the effect of age on the activity of the pathway was not known at this stage. All participants were considered healthy based on the measurements in Table 3.1 and information given in screening questionnaires. Males and females did not significantly differ by age, BMI, plasma total cholesterol concentration or plasma glucose concentration. Females had significantly higher Plasma HDL concentration (P < 0.0001). As expected mean weight and total haemoglobin were significantly lower in females (P < 0.001) and body fat percentage significantly higher than in men (P < 0.001). The proportions of T-lymphocytes (CD3+), B-lymphocytes (CD19+) and monocytes (CD14+) within the PBMC population are shown for freshly isolated (pre-culture) and cultured (post-culture) PBMCs with and without Con A stimulation, in Table 3.2. There was a significant increase in the proportion of T-lymphocytes (P < 0.01) and a significant decrease in the proportion of monocytes (P < 0.0001) following both unstimulated and stimulated culture compared with freshly isolated cells. The proportion of B-lymphocytes also significantly decreased following culture under stimulating conditions (P < 0.01), compared with freshly isolated cells.
Table 3.1. Characteristics of population used for unstimulated and stimulated PBMC comparisons

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male</th>
<th>Female</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>30 [18-38]</td>
<td>34 [20-47]</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>78.8 ± 2.3</td>
<td>66.9 ± 2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 0.6</td>
<td>23.7 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>19.3 ± 1.2</td>
<td>30.8 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma TG (mmol/l)</td>
<td>1.2 ± 0.09</td>
<td>0.9 ± 0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma total cholesterol (mmol/l)</td>
<td>4.5 ± 0.3</td>
<td>4.6 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma HDL cholesterol (mmol/l)</td>
<td>1.1 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>4.9 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Total haemoglobin (g/l)</td>
<td>146 ± 3.0</td>
<td>122 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean ± SEM; * data are mean [range]; Male n = 16, Female n = 18. P values are for differences between females and males determined using an independent samples t-test.

Table 3.2. PBMC phenotypes

<table>
<thead>
<tr>
<th>Cell phenotype (% of PBMCs)</th>
<th>Pre-culture</th>
<th>Post-culture 0 µg/ml Con A</th>
<th>Post-culture 5 µg/ml Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>62.7 ± 1.6a</td>
<td>69.8 ± 1.7b</td>
<td>70.5 ± 1.8b</td>
</tr>
<tr>
<td>CD19+</td>
<td>7.5 ± 0.5a</td>
<td>5.8 ± 0.5ab</td>
<td>5.6 ± 0.4b</td>
</tr>
<tr>
<td>CD14+</td>
<td>11.4 ± 0.9a</td>
<td>3.2 ± 0.3b</td>
<td>1.6 ± 0.3b</td>
</tr>
</tbody>
</table>

Data are mean ± SEM; n = 34. Results are for positive events within the PBMC gate, measured by flow cytometry. Statistical comparisons were by one-way ANOVA with Tukey’s post hoc test. For each cell phenotype, means that were significantly different between groups are indicated by different superscripts (P < 0.01).

3.3.2 The effect of 18:3n-3 treatment on viability and proliferation

Since measurement of n-3 PUFA synthesis requires addition of stable isotope labelled 18:3n-3 to the culture medium, the effect of 18:3n-3 supplementation on PBMC viability and proliferation was determined using purchased PBMCs. The proportion of live cells in unstimulated PBMC cultures was not affected by treatment with any concentration of 18:3n-3 tested (Figure 3.2) but was significantly decreased in Con A stimulated cells treated with 50 µM 18:3n-3 (P < 0.0001). The number of viable PBMCs assessed by cellular ATP content (Figure 3.3) was not affected by any 18:3n-3 concentration tested in unstimulated cultures but was significantly decreased by all three concentrations of 18:3n-3 (P < 0.0001) in Con A stimulated cultures. Treatment of Con A stimulated...
PBMC cultures with 20 µM 18:3n-3 significantly decreased all CFSE proliferative measures (Figure 3.4): % divided (P < 0.01), division index (P < 0.001) and proliferation index (P < 0.0001).

Figure 3.2. The effect of 18:3n-3 treatment on PBMC viability.
The proportion of live cells was determined for total cell events using the LIVE/DEAD viability dye and flow cytometry. Values are mean ± SEM (n = 6 replicates per treatment group) for unstimulated (no Con A) and stimulated (+ Con A) cultures. 18:3n-3 (µM) indicates final concentration in culture medium. Statistical analysis was by One-Way ANOVA with Dunnett’s post hoc test. ****P < 0.0001 compared to the control group (0 µM 18:3n-3).

Figure 3.3. The effect of 18:3n-3 treatment on relative ATP content of PBMCs.
Relative ATP content of PBMCs was determined using the CellTiter-Glo Luminescent cell viability assay. 18:3n-3 (µM) indicates final concentration in culture medium. Values are mean ± SEM (n = 6 replicates per treatment group) for unstimulated (no Con A) and stimulated (+ Con A) cultures. Statistical analysis was by One-Way ANOVA with Dunnett’s post hoc test. ****P < 0.0001 compared to the control group (0 µM 18:3n-3).
Figure 3.4. The effect of 18:3n-3 on proliferation in stimulated PBMCs.

Graphs A (% Divided), B (Division Index) and C (Proliferation Index) show results for gated events with the light scatter characteristics of viable lymphocytes following CFSE staining. 18:3n-3 (µM) indicates final concentration in culture medium. Values are mean ± SEM (n = 6 replicates per treatment group) Statistical analysis was by independent-samples t-test. ****P < 0.0001, ***P < 0.001, **P < 0.01.

3.3.3 The effect of PBMC activation on CD69 expression

For unstimulated versus stimulated comparisons, first activation by Con A was confirmed by measuring CD69 expression on the cell surface (Figure 3.5). Treatment of PBMCs with 5 µg/ml Con A increased the proportion of CD69 positive events in all samples, compared with untreated cells and the mean increase was statistically significant (P < 0.0001). Statistical analysis of separate male and female groups, by paired-samples t-test (data not shown), revealed that Con A treatment significantly increased CD69 expression in both males and females (P < 0.0001).

Figure 3.5. The effect of PBMC activation on CD69 expression.

Graphs show results for gated events with the light scattering characteristics of PBMCs (n = 34). Left: % CD69+ events for unstimulated (0 µg/ml Con A) and Con A stimulated (5 µg/ml Con A) PBMCs. Right: Difference in % CD69+ events (stimulated minus unstimulated) showing mean ± SEM. Statistical analysis was by paired-samples t-test (P value is shown on the right hand graph).
3.3.4 The effect of PBMC activation on n-3 PUFA synthesis

Stable isotope enrichment in [1-\textsuperscript{13}C]18:3n-3 conversion products confirmed de novo synthesis of individual n-3 PUFAs in PBMCs, except for 22:6n-3 where there was no enrichment and 18:4n-3 where there was no detectable fatty acid peak. Increased activity of the PUFA synthesis pathway was detected in Con A stimulated cells. The level of [1-\textsuperscript{13}C]18:3n-3 was significantly higher in stimulated cells (P < 0.0001) and the levels of its stable isotope labelled conversion products 20:3n-3 (P < 0.0001), 20:4n-3 (P < 0.0001), 20:5n-3 (P < 0.001) and 22:5n-3 (P < 0.0001) were also significantly higher (Figure 3.6). Analysis of male and female groups separately (data not shown), revealed significant increases in 18:3n-3, 20:3n-3, 20:4n-3, 22:5n-3 (P < 0.001 for all) and 20:5n-3 (P < 0.05) in Con A treated PBMCs in both males and females.

3.3.5 Relationship between CD69 expression and n-3 PUFA synthesis

The relationships between the amounts of labelled n-3 PUFAs and CD69 positive events within the PBMC gate were analysed by Spearman’s rank-order correlation (Figure 3.7). This analysis was carried out on the total EPUFA study population (characteristics are given in Table 7.1, Chapter 7). The sum of the 18:3n-3 conversion products 20:3n-3, 20:4n-3, 20:5n-3 and 22:5n-3 was calculated to determine the relationship between total n-3 PUFA synthesis and CD69 expression. Uptake of 18:3n-3 showed a significant positive correlation with CD69 expression (R_s = 0.257, P < 0.05). CD69 expression was also correlated positively with the amount of [\textsuperscript{13}C]20:3n-3 (R_s = 0.483, P < 0.001), [\textsuperscript{13}C]20:4n-3 (R_s = 0.392, P < 0.01), [\textsuperscript{13}C]20:5n-3 (R_s = 0.531, P < 0.0001), [\textsuperscript{13}C]22:5n-3 (R_s = 0.555, P < 0.0001) and total synthesised [\textsuperscript{13}C]n-3 PUFA (R_s = 0.534, P < 0.0001). The strongest relationships existed for 22:5n-3 and total synthesised n-3 PUFA.
Figure 3.6. The effect of PBMC activation on n-3 PUFA synthesis.

**Left:** $[^{13}C]$-labelled n-3 PUFAs in unstimulated (0 µg/ml Con A) and Con A stimulated (5 µg/ml Con A) PBMCs. **Right:** Difference in $[^{13}C]$-labelled n-3 PUFAs (stimulated minus unstimulated) showing median ± IQR (n = 32). Statistical analysis was by Wilcoxon signed-rank test (P value is shown on the right hand graph).
Figure 3.7. Relationship between n-3 PUFA synthesis and CD69 expression.
The relationships between uptake of 18:3n-3 and synthesis of individual n-3 PUFAs and total synthesised n-3 PUFAs and CD69 expression were assessed by Spearman’s rank-order correlation, where $r_s$ is the Spearman’s Correlation coefficient.

3.3.6 The effect of delta-6 desaturase inhibition on T-lymphocyte proliferation

The effect of SC-26196 treatment on proliferation was measured using the intracellular stain CFSE at five time points. Proliferation was measured for events with the light scattering characteristics
of viable lymphocytes and since Con A is a T-lymphocyte mitogen it is assumed that the analysis is representative of this cell type.

The effect of treatment with 50 nM, 100 nM and 200 nM SC-26196 on the viability of PBMCs cultured with 5 µg/ml ConA was determined using the LIVE/DEAD red viability dye (Figure 3.8). Viability for the vehicle control group was 79.8 %. Viability was significantly reduced to 77.9 % (P < 0.01) 76.9 % (P < 0.001) and 77.4 % (P < 0.01) for the 50 nM, 100 nM and 200 nM SC-26196 groups, respectively. Inhibition of delta-6 desaturase activity by SC-26196 was confirmed by measuring conversion of [1-13C]18:3n-3 in a T-lymphocyte cell line (section 6.3.4, Chapter 6).

Con A treated cells showed multiple CFSE fluorescence peaks over time indicating multiple generations of proliferating cells, where unstimulated cells showed a single bright fluorescence peak indicating no activation (Figure 3.9). Three measures of proliferation were recorded: % divided, division index and proliferation index (Figure 3.10). Briefly, % divided represents the percentage of the original cell population that entered cell division, the division index represents the average number of divisions for the entire population and the proliferation index is a measure of the average number of divisions for the responding population (see section 2.10.3 for more details). Statistical analysis by two-way repeated measures ANOVA reported a significant interaction between time and SC-26196 concentration for each measure: proliferation index (P < 0.01, partial η² = 0.42), division index (P < 0.0001, partial η² = 0.56) and % divided (P < 0.01, partial η² = 0.40). Since there were significant interactions, simple main effects were analysed. Treatment with 200 nM SC-26196 significantly decreased the percentage of cells that had entered division (% divided) after 72 h (P < 0.01) and 96 h (P < 0.05), compared to the control group. The division index was significantly decreased by treatment with 200 nM SC-26196 after 72 h, 96 h and 144 h culture (P < 0.01 for all) and by treatment with 100 nM SC26196 after 144 h culture (P < 0.01), compared to the control group. After 144 h culture the proliferation index was significantly decreased by treatment with 100 nM and 200 nM SC-26196 compared to the control group (P < 0.01 for both). There was no effect of SC-26196 treatment on the proliferation index, division or percentage of dividing cells after 120 h culture.
Figure 3.8. The effect of SC-26196 treatment on PBMC viability.
The proportion of live cells was determined for total cell events using the LIVE/DEAD red viability dye and flow cytometry. Values are mean ± SEM (n = 6 for each treatment group). Statistical analysis was by one-Way ANOVA with Dunnett’s post hoc test for comparison of each SC-26196 concentration to the vehicle control (0 nM SC-26196). ***P < 0.001, **P < 0.01.

Figure 3.9. Histograms showing proliferation of CFSE stained T-lymphocytes over time.
The graphs show results for gated events with the light scatter characteristics of viable lymphocytes. The red line shows undivided cells (cells cultured in the absence of Con A) and the blue line shows cells cultured in the presence of 5 µg/ml Con A.
Figure 3.10. The effect of SC-26196 on proliferation of T-lymphocytes activated by Con A.

Graphs A (% Divided), B (Division Index) and C (Proliferation Index) show results for gated events with the light scatter characteristics of viable lymphocytes following CFSE staining. Values are mean ± SEM. Statistical analysis was by two-way repeated measures ANOVA with time and SC-26196 concentration as fixed factors. Simple main effects were analysed to compare treatment with 100 nM SC-26196 (green stars) and 200 nM SC-26196 (blue stars) to the 0 nM SC-26196 control at each time-point, **P < 0.001, *P < 0.05.
3.4 Discussion

Overall, the findings from these experiments confirmed that activation is required to stimulate n-3 PUFA synthesis in PBMCs.

Characterisation of cell phenotypes using flow cytometry confirmed successful extraction of PBMCs from participants’ blood samples. In humans, the frequencies of these populations vary across individuals, however, the proportions of CD3+ T-lymphocytes, CD19+ B-lymphocytes and CD14+ monocytes in PBMCs (Table 3.2) were consistent with previously reported proportions in healthy individuals (276). The culture environment significantly increased the proportion of T-lymphocytes in the PBMC population compared to freshly isolated cells. However, there was no significant difference in the proportion of T-lymphocytes between stimulated and unstimulated cultures, which is surprising since Con A stimulates T-lymphocytes to proliferate. A possible explanation could be down modulation of the TCR-CD3 complex, which has been reported to occur following activation (277-279). Previous findings indicate that activation causes down modulation by preventing recycling rather than inducing internalisation of the TCR-CD3 complex (280, 281). A consequence of this possible down-modulation would be underestimation of T-lymphocytes. Co-staining with antibodies for CD3 and other markers for T-lymphocyte sub-populations such as CD4 and CD8 would be necessary to overcome this limitation and give greater confidence in accurately identifying cells. There was a small yet significant reduction in the proportion of B-lymphocytes following culture with Con A (5.6%) compared to freshly isolated PBMCs (7.5%). A larger decrease was observed for monocytes, which decreased from 11.4% in freshly isolated cells to 3.2% and 1.6% after culture in the presence and absence of Con A, respectively. This decrease was probably due to the adherent nature of monocytes and consequently the challenge in recovering them.

Differential staining of live and dead cells showed that treatment of Con A stimulated PBMCs with 10 µM or 20 µM 18:3n-3 did not decrease the proportion of viable cells in the population, however there was a significant reduction in cell viability with 50 µM 18:3n-3 treatment as shown in Figure 3.2. In contrast, the viability of unstimulated PBMCs was not affected by any of the 18:3n-3 concentrations tested. Viability was also assessed using an assay that determined the number of viable cells in culture based on quantitation of ATP and therefore the result reflected the ability of the cells to proliferate, not merely their survival. In unstimulated PBMCs, 18:3n-3 treatment did not affect ATP content relative to the vehicle control. Since unstimulated PBMCs do not proliferate in culture this result was interpreted as 18:3n-3 having no effect on cell survival, supporting the results of the viability staining. However, in cells stimulated to proliferate, the number of viable cells relative to the control was significantly lower at all 18:3n-3 concentrations tested indicating that 18:3n-3 treatments affects the ability of cells to proliferate in culture. This was confirmed by CFSE
staining in cells cultured for a longer period (120 h) where all measured proliferative indices were significantly lower in PBMCs cultured in the presence of 20 µM 18:3n-3. This demonstrates that the system is behaving as previously shown, where the proliferative response to Con A treatment was inhibited by 18:3n-3 (164). Supplementation of culture medium with a concentration of 20 µM stable isotope labelled 18:3n-3 was chosen for subsequent measurement of n-3 PUFA synthesis as it did not reduce cell survival and although this concentration had a small inhibitory effect on proliferation it was required for detection of fatty acid peaks for isotopic analysis.

Con A treatment caused a marked increase in the proportion of cells expressing the activation marker CD69. CD69+ events increased from a mean of 1.4% in unstimulated cells to 51.2% in stimulated cells, confirming cell activation. This level is 10% lower than reported in a study by other researchers where lymphocytes were treated with 5 µg/ml Con A (265). However, they measured CD69 expression in CD3+CD4+ T-lymphocytes after 24 h culture compared to the total PBMC population after 48 h in the present study. Since Con A is an activator of T-lymphocytes, measurement of CD69 positive cells within the T-lymphocyte population would explain the higher proportion observed. CD69 is an early activation marker and expression peaks by 24 h and then declines (282). A decrease in CD69 expression in CD3+ T-lymphocytes by approximately 10% between 15 and 48 h of culture, dramatically dropping by 50% between 15 and 96 h has been reported in PHA stimulated lymphocytes (283). Therefore, measurement of CD69 after 48 h in this study allowed confirmation of cell activation but may have not been within the optimal time-period for measurement. However, in other studies it was found that the maximum number of CD3+ cells expressing CD69 occurred by 24 h stimulation with Con A and remained stable for a 72 h culture period (284) or reduced slightly (285).

The level of stable isotope labelled 18:3n-3 was significantly higher in Con A treated PBMCs (Figure 3.6) indicating that activation increases the uptake of 18:3n-3 from the culture medium, which is consistent with other studies that have shown that media supplementation with 18:3n-3 increases its proportion in stimulated cells (49, 147). The levels of labelled 18:3n-3 conversion products were also significantly higher in activated cells, except for 22:6n-3 where there was no evidence for synthesis. These results are in agreement with the single previous study that showed activation of the n-3 PUFA conversion pathway in stimulated cells (147). However, the previous study did not distinguish individual n-3 PUFAs and instead reported results for groups of n-3 PUFAs based on the number of double bonds in the carbon chain. Therefore, this is the first time that significant increases in the synthesis of individual fatty acids in the n-3 PUFA pathway, up to 22:5n-3, have been shown in stimulated compared with unstimulated PBMCs.
The major metabolite of 18:3n-3 after 48 h was 20:3n-3, which is a product in the alternative delta-8 desaturation pathway. However, the data for 20:3n-3 synthesis contained an outlier with a [1-13C]20:3n-3 value of 2.91 pmol/µg protein compared to 1.31 pmol/µg protein for the next highest value. Removal of this outlier would make 20:3n-3 synthesis appear more comparable with 20:4n-3 synthesis. However, these data were analysed by Wilcoxon signed-rank test, a non-parametric test, which tested the null hypothesis that the median difference in n-3 PUFA synthesis between unstimulated and Con A stimulated PBMCs is equal to zero. Since data are ranked, the outlier would simply be recognised as a case that is ranked one above the case below and therefore this test is resistant to outliers. Nevertheless, whether this outlier is a true result is an important question and would need to be assessed by repeating the experiment, which was not possible due to limited sample.

Measurement of 20:3n-3 synthesis in activated PBMCs in the present study, supports the findings from a previous study on n-3 PUFA synthesis in PHA stimulated PBMCs, where media supplementation with unlabelled 18:3n-3 significantly increased the proportion of 20:3n-3 but did not significantly increase the proportions of any other n-3 PUFAs (147). It is important to highlight that this previous study did not measure stable isotope labelled 20:3n-3 and therefore the findings from this chapter provide the first direct evidence for synthesis of 20:3n-3 in mitogen treated human PBMCs.

The first product in the classical pathway (18:4n-3) could not be detected indicating that activated PBMCs may be using the alternative pathway as the major route for n-3 PUFA synthesis. A delta-8 desaturation step would be required to feed 20:3n-3 back into the main pathway. The occurrence and importance of delta-8 desaturation in mammalian tissues has been a debateable issue. Previous studies have provided evidence for delta-8 desaturation in rat testes (38), human testes (39) and also in mouse rat and human cultured cell lines (41, 42). Contradictory to these findings, some experiments in mice liver and rat liver and testes have reported delta-8 desaturation as either not occurring or as a questionable route for the biosynthesis of PUFAs (33, 36, 37). Furthermore, there is no molecular evidence for a specific gene that encodes delta-8 desaturase activity. A study in yeast transformed with mammalian FADS2 demonstrated that the delta-6 desaturase enzyme encoded by FADS2 had the ability to catalyse delta-8 desaturation of 20:3n-3 and 20:2n-6 to yield 20:4n-3 and 20:3n-6, respectively (19). However, delta-8 desaturation appeared to be a minor route for n-3 PUFA synthesis, since delta-6 desaturase activity was shown to be favoured 23-fold over delta-8 desaturase activity. The biological importance of this alternative pathway is not known. Possible explanations could include providing a route for n-3 and n-6 PUFA biosynthesis if the classical pathway is inhibited. However, if the delta-8 desaturation step is catalysed by the product of FADS2, inhibition of ‘delta-6 desaturase’ would also inhibit conversion of 20:3n-3 to 20:4n-3,
unless there is another desaturase that is capable of catalysing this step. It has been suggested that the alternative pathway could be important in tissues with a high demand for eicosanoid synthesis, since delta-8 desaturation of 20:2n-6 (the n-6 equivalent to 20:3n-3 in the alternative delta-8 desaturation pathway) directly yields 20:3n-6, which is an immediate precursor of prostaglandin E₁ (PGE₁) (19). This may not be relevant in lymphocytes as a review of studies investigating eicosanoid synthesis concluded lymphocytes have poor capacity for synthesis of eicosanoids (168). Nonetheless, monocytes present in PBMC populations do possess the capacity for eicosanoid synthesis and an earlier study demonstrated PUFA synthesised by lymphocytes can be utilised by monocytes for eicosanoid synthesis (286). At present, there is no evidence that 20:3n-3 and 20:2n-6, or their delta-5 desaturation conversion products 20:4(5,11,14,17) and 20:3(5,11,14), respectively, are metabolised directly to lipid signalling molecules. However, 20:2n-6 has been shown to modulate the production of inflammatory mediators, including increasing prostaglandin E₂ (PGE₂) and tumour necrosis factor-α (TNF-α) production, in murine macrophages (287). Furthermore, in another study, 20:2n-6 was found to be a potent inhibitor of leukotriene B₄ (LTB₄) binding to pig neutrophil LTB₄ receptors (288). Therefore, given the level of 20:3n-3 synthesis measured in the present work, it would be interesting to investigate the effect of this fatty acid on inflammatory mediators in future experiments. A limitation of the EPUFA study design was that participants potentially taking anti-inflammatory agents such as aspirin were not excluded from the study. Aspirin can trigger the synthesis of 20:5n-3 and 22:6n-3 derived inflammatory mediators called resolvins; several epimeric isoforms are generated as a result of the effects of aspirin on the activity of COX-2. Consequently, consumption of aspirin may have influenced the levels of 20:5n-3 and 22:6n-3 measured in PBMCs.

There were significant positive correlations between all the newly synthesised n-3 PUFAs that were measured and the cell surface expression of CD69 in PBMCs (Figure 3.7). This further supports the finding that n-3 PUFA synthesis is increased in stimulated PBMCs and suggests the level of synthesis relates to the number of activated cells. The strongest correlations were for 22:5n-3 and the sum of all synthesised n-3 PUFA. The sum was calculated to give a measurement of overall synthesis since the amount of individual fatty acids does not only reflect their synthesis but also their conversion. Whether n-3 PUFA synthesis precedes or succeeds CD69 expression or whether there is causal relationship between them is unknown. The signals necessary for appropriate CD69 regulation appear to be within a 700 bp region of the start of transcription of the gene (289). The regulation of many genes involved in T-lymphocyte activation occurs largely at the transcriptional level (290). Since n-3 PUFAs are known regulators of gene transcription it would be interesting to investigate whether they play a role in the signal transduction events leading to induction of CD69 expression.
Analysis of cell proliferation using the intracellular dye CFSE enabled characterisation of the response of Con A stimulated T-lymphocytes within a PBMC population to treatment with the delta-6 desaturase inhibitor SC-26196. Proliferation analysis was performed for events within the lymphocyte gate but since Con A is a T-lymphocyte mitogen it was assumed that the analysis was representative of this lymphocyte sub-set. Con A treatment induced multiple CFSE fluorescence peaks that increased over time indicating multiple generations of proliferating T-lymphocytes (Figure 3.9). Unstimulated cells showed a single bright fluorescence peak confirming no proliferation. Inhibition of delta-6 desaturase activity decreased both the proportion of cells that entered into cell division and the number of cell divisions in the total and responding (cells that entered division) cell populations after 144 h culture. These indices of proliferation were also significantly lower at earlier time points when cells were treated with the higher (200 nM) concentration of SC-26196. However, there were no differences after 120 h culture. Despite the statistically significant differences, the effects were small and therefore biological significance is questionable. Furthermore, treatment with all SC-26196 concentrations was associated with a small (< 3%) but significant decrease in PBMC viability relative to the vehicle control. This study showed that there was considerable variability in capacity for n-3 PUFA synthesis between different individuals (Figure 3.6). Therefore, the results from this inhibitor experiment might be explained by the fact that delta-6 desaturase activity in PBMCs from this individual was negligible. This is a limitation when using purchased PBMCs from a single individual and repeating the experiment with cells from individuals with a range of synthesis capacities would provide more insight into how the pathway activity relates to proliferation. Furthermore, the concentrations of SC-26196 used in this experiment did not completely restrict PUFA synthesis and it could be that enough PUFA synthesis was sustained for proliferation. Nonetheless, these results suggest that inhibition of the pathway may be having a small effect on the proliferative process. This supports the proposed requirement of n-3 PUFA synthesis in fine-tuning membrane fatty acid composition needed to support membrane synthesis in proliferating lymphocytes (147).

The suggestion that n-3 PUFA synthesis is required for proliferation conflicts the evidence that they inhibit proliferation. However, this could be explained by the concentration of fatty acids used as lower concentrations of some unsaturated fatty acids have been shown to increase lymphocyte proliferation (167). Alternatively, it could be that n-3 PUFA synthesis functions in modulating the proliferative response. The action of n-3 PUFAs are complex and as discussed in section 1.10, they are reported to have numerous and diverse immunomodulatory effects. For example, by influencing the function of membrane associated proteins and signaling pathways through their effect on membrane fluidity, by modulating gene expression including the mRNA expression of
cytokine genes and through modifying the production and activity of inflammatory mediators such as eicosanoids and resolvins.

In summary, treatment of PBMCs with the T-lymphocyte mitogen Con A significantly increased the expression of the activation marker CD69, the uptake of 18:3n-3 and synthesis of its conversion products 20:3n-3, 20:4n-3, 20:5n-3 and 22:5n-3 compared with untreated PBMCs, where synthesis was negligible. The most highly synthesised n-3 PUFA was 20:3n-3, a product in the alternative delta-8 desaturation pathway, which increased 10-fold in activated cells. Up-regulation of the pathway in stimulated cells suggests PUFA synthesis may have an important role in the activation and/or function of proliferating lymphocytes. However, reducing PUFA synthesis using a delta-6 desaturase inhibitor only had a small effect on the proliferative response. Consequently, whether PUFA synthesis is needed for lymphocyte activation and proliferation is questionable and a complete knockdown of PUFA synthesis in future experiments would be required to answer this question.
Chapter 4: Does activation of PBMCs change transcriptional regulation of the genes encoding the desaturase and elongase enzymes involved in the PUFA biosynthesis pathway?
4.1 Introduction

Activation of T-lymphocytes results in an elaborate transcriptional response involving increased expression of a multitude of genes that function in cell proliferation and immune function (290, 291). This transcriptional response has been shown to include increased expression of genes involved in fatty acid uptake and biosynthesis in activated CD4+ T-lymphocytes (146).

The findings reported in Chapter 3 showed that n-3 PUFA synthesis significantly increased in Con A activated PBMCs compared with untreated cells. This confirmed previous findings where n-3 PUFA synthesis increased in PBMCs stimulated with PHA (147). Previous studies in rat liver and the HepG2 liver cancer cell line have shown associations between n-3 and n-6 PUFA synthesis levels and the transcriptional activity of the genes encoding the pathway enzymes (104, 105, 213). However, it is not known whether increased activity of the pathway in activated PBMCs is associated with higher mRNA expression of the genes encoding the enzymes involved in the main PUFA biosynthesis pathway: delta-6 desaturase (FADS2), delta-5 desaturase (FADS1), elongase-5 (ELOVL5) and elongase-2 (ELOVL2). The identities of the enzymes that operate in the alternative delta-8 desaturation pathway, shown to be active in PBMCs, are not clear. Experiments involving heterologous expression of ELOVL5 and FADS2 in S. cerevisiae have provided evidence that the ELOVL5 gene product is capable of elongating 18:3n-3 to 20:3n-3 (26) and the FADS2 gene product is capable of desaturating 20:3n-3 at the delta-8 position to yield 20:4n-3 (19). However, it is not known if these processes occur in humans.

Aim

To investigate the effect of PBMC activation on the mRNA expression of the desaturase and elongase enzymes involved in the PUFA synthesis pathway.

Hypotheses

- **FADS2, FADS1, ELOVL5 and ELOVL2** mRNA expression levels are higher in response to PBMC activation.
- **FADS2, FADS1, ELOVL5 and ELOVL2** mRNA expression levels in activated PBMCs are positively correlated with the proportion of activated cells.

To address these hypotheses, the relative mRNA expression of **FADS2, FADS1, ELOVL5** and **ELOVL2** were compared between PBMCs cultured with and without the T-lymphocyte mitogen Con A and the relationships between the expression of these genes and the proportion of Con A treated PBMCs expressing CD69 were analysed.
4.2 Methods

All PBMC samples were obtained from volunteers participating in the EPUFA study described in section 2.2. PBMCs were isolated and cultured in the presence or absence of Con A using the experimental design described in section 3.2 and summarised in Figure 3.1 (Chapter 3). The subject characteristics and PBMC phenotypes are given in Table 3.1 and Table 3.2, respectively.

4.2.1 Measurement of mRNA expression

The overall procedure for measuring mRNA expression by real-time RT-PCR is given in section 2.15. Briefly, total RNA was extracted from PBMC pellets and converted to cDNA. cDNA was amplified by real-time RT-PCR using commercially prepared primer pairs for FADS2, FADS1, ELOVL5 and ELOVL2. Quantitation of mRNA expression levels was carried out using the relative standard curve method described in section 2.15.3.1 with normalisation to four reference genes (EIF4A2, RPL13A, SDHA and 18S rRNA). Reference genes were selected based on expression stability determined by GeNorm analysis (2.15.3.2). The amplification efficiency of each real-time RT-PCR assay was calculated from the slope of the standard curve using the equation below.

Amplification efficiency = \[10^{(-1/\text{slope})}}-1\]
4.3 Results

PCR efficiency, evaluated from the slope of each standard curve, was 96% for FADS2, 94% for FADS1 and 98% for both ELOVL5 and ELOVL2 assays. Standard curves for all primer sets had an R² value > 0.99 (Figure 4.1). ELOVL2 was below the lower limit of quantification for all unstimulated samples and was quantifiable in 12 out 34 Con A stimulated samples. Furthermore, for 21 unstimulated PBMC samples and 6 stimulated PBMC samples, no amplification above background fluorescence was detected. Consequently, relative ELOVL2 mRNA expression between unstimulated and stimulated samples could not be analysed. Con A treatment significantly increased the mRNA expression of FADS2 (P < 0.0001), FADS1 (P < 0.01) and ELOVL5 (P < 0.01) compared with untreated cells (Figure 4.2). The differences between mRNA expression levels in Con A stimulated and unstimulated PBMCs are shown in Figure 4.2 (right), where positive values represent an increase in mRNA expression, negative values a decrease and zero represents no expression change. The median difference in FADS2, FADS1 and ELOVL5 relative mRNA expression between Con A stimulated and unstimulated PBMCs was 0.26, 0.04 and 0.51, respectively. Statistical analysis by Wilcoxon signed-rank test of separate male and female groups (data not shown) revealed that Con A treatment significantly increased FADS2 mRNA expression in both males (P < 0.0001) and females (P < 0.01) compared with untreated cells, whereas FADS1 (P < 0.05) and ELOVL5 (P < 0.05) were also significantly increased in males.
Figure 4.1. Real-time RT-PCR standard curves for FADS2, FADS1, ELOVL5 and ELOVL2 assays.
Figure 4.2. The effect of PBMC activation on FADS2, FADS1 and ELOVL5 mRNA expression.
Left: mRNA expression normalised to EIF4A2, 18S, SDHA and RPL13A for unstimulated (0 µg/ml Con A) and stimulated (5 µg/ml Con A) PBMCs. Right: Difference scores in mRNA expression (Con A stimulated minus unstimulated) showing median and IQR. Statistical analysis was by Wilcoxon signed-rank test (P value is shown on the right hand graph) for FADS2 (n = 32), FADS1 (n = 31) and ELOVL5 (n = 28).
4.3.1 Relationship between CD69 expression and mRNA expression

The relationship between the proportion of CD69 positive events measured in the PBMC gate by flow cytometry and relative mRNA expression were analysed by Spearman’s rank-order correlation for the total EPUFA population (Figure 4.3). There were significant positive correlations between CD69 expression and the mRNA expression of FADS2 ($r_s = 0.354, P < 0.01$), FADS1 ($r_s = 0.470, P < 0.0001$) and ELOVL5 ($r_s = 0.348, P < 0.01$).

![Figure 4.3. Relationship between CD69 expression and FADS2, FADS1 and ELOVL5 mRNA expression.](image)

The relationships between the proportion of CD69 positive events (％CD69+) measured within a PBMC gate and the relative mRNA expression of FADS2 (n = 67), FADS1 (n = 66) and ELOVL5 (n = 66) were assessed by Spearman’s rank-order correlation, where $r_s$ is the Spearman’s Correlation coefficient.
4.4 Discussion

Activation of PBMCs by Con A significantly increased the mRNA expression of three genes (FADS2, FADS1, and ELOVL5) encoding enzymes involved in conversion of 18:3n-3 to longer chain metabolites, compared with untreated PBMCs. In Chapter 3, it was shown that the synthesis of 20:3n-3, 20:4n-3, 20:5n-3 and 22:5n-3 was also significantly increased in Con A activated cells. These findings suggest that changes in the mRNA expression of these genes affects the capacity of cells for n-3 PUFA synthesis. This is in agreement with other studies that have shown that increased activity of this PUFA biosynthesis pathway is associated with increased mRNA expression of the genes encoding the enzymes that operate in the pathway (105, 213). Analysis of the proportion of cells within the PBMC gate expressing the cell surface activation marker CD69 revealed significant positive correlations between CD69 expression and the mRNA expression of FADS2, FADS1, and ELOVL5. This suggests that the level of transcription relates to the level of cell activation and complements the positive relationships observed between the proportion of cells expressing CD69 and the levels of synthesised n-3 PUFAs. Taken together, the present findings suggest that the effects of Con A activation on n-3 PUFA synthesis are mediated at the transcriptional level.

Consistent with the current findings, upregulation of FADS2 mRNA expression in activated lymphocytes has been demonstrated previously. Antibody induced activation of cultured CD4+ T-cells increased FADS2 mRNA expression compared with naïve CD4+ T-cells, which was shown to be controlled by direct binding of the transcription factor SREBP1 activated through TCR stimulation and MTOR-mediated signalling (146). However, in contrast to the present study, Angela et al., 2016 found no increase in ELOVL5 mRNA expression. It is presumed that the present analysis should be most representative of T-lymphocyte activation since the majority of PBMCs are T-lymphocytes and Con A primarily activates this lymphocyte subset. However, the difference in findings could be explained by ELOVL5 mRNA expression changing in a different cell type or lymphocyte subpopulation present in PBMCs. PBMCs are a heterogeneous population of cells and therefore measured gene expression profiles are not resolved to an individual cell population; a limitation when interpreting gene expression results as it is not known which cell types are the source of detected differences.

Chapter 3 reported no measurable fatty acid peak for 18:4n-3, the delta-6 desaturase conversion product of 18:3n-3 and the most highly synthesised n-3 PUFA was 20:3n-3, which is the immediate elongation product of 18:3n-3. Together, this suggests that PBMCs may use the alternative delta-8 desaturation pathway as the primary route to longer chain n-3 PUFA synthesis. However, FADS2 mRNA was expressed in PBMCs and increased in activated cells. This suggests conversion of 18:3n-3 to 18:4n-3 could have been operating but the presence of FADS2 protein would need to be
verified by Western blotting. Inspection of raw Ct values suggested that ELOVL5 (mean Ct 23.53), was more highly expressed than FADS2 (mean Ct 26.86); based on the principle that the lower the RT-PCR template amount, the more amplification cycles are needed to reach the threshold fluorescence. However, this should be interpreted with caution as the amplification efficiency was not identical for the FADS2 (96%) and ELOVL5 (98%). In a previous study, expression of human ELOVL5 in S. cervisiae revealed that it is capable of elongating 18:3n-3 to 20:3n-3 (26). Therefore, since in the present study both ELOVL5 expression and 20:3n-3 synthesis were elevated, it is speculated that elongation of 18:3n-3 to 20:3n-3 in PBMCs is catalysed by elongase-5. Furthermore, potential competition between elongase-5 and delta-6 desaturase for the substrate 18:3n-3 may explain the predominance of the alternative delta-8 desaturation pathway. However, the involvement of elongase-5 in 20:3n-3 synthesis in PBMCs would need confirming, which would be possible by using siRNA to knock down ELOVL5 expression.

ELOVL2 mRNA expression was below the quantitation limit of the assay for all the unstimulated samples and was only quantifiable in four of the 34 stimulated samples. Furthermore, no amplification of ELOVL2 could be detected in 21 of the unstimulated compared with six of the Con A stimulated PBMC samples. This indicates that the mRNA expression of ELOVL2 is either absent or very low in both unstimulated and stimulated PBMCs. The observation that amplification of ELOVL2 was detectable in a greater number of stimulated compared with unstimulated samples suggests some upregulation of ELOVL2 in stimulated cells may occur. However, the biological importance of very low copy numbers of ELOVL2 mRNA is not known. Elongase-2 has been shown to be involved in the conversion of 22:5n-3 to 22:6n-3 by elongation of 22:5n-3 to the 24 carbon intermediate 24:5n-3 (27). Consequently, the minimal expression of ELOVL2 mRNA could explain the lack of 22:6n-3 synthesis observed in PBMCs. However, conversion of 22:5n-3 to 22:6n-3 involves multiple steps including desaturation at the delta-6 position, translocation to peroxisomes and one cycle of β-oxidation, after which 22:6n-3 may be further degraded or moved back to the endoplasmic reticulum for membrane biosynthesis (4). Therefore, there are additional steps that could act as control points in the synthesis of 22:6n-3 and incubation of PBMCs with the pathway intermediates downstream of elongation (24:5n-3) and desaturation (24:6n-3) could help to verify the mechanism responsible. Finally, it has been shown that conversion of 20:5n-3 to 22:5n-3 can be catalysed by both elongase-2 and elongase-5 enzymes (29) but the minimal expression of ELOVL2 mRNA suggests that ELOVL5 may be more important for the conversion of 20:5n-3 to 22:5n-3 in activated PBMCs.

In summary, Con A mediated activation of PBMCs significantly increased the mRNA expression of FADS2, FADS1 and ELOVL5 compared with untreated cells. ELOVL2 expression was below the detection limit and may explain the lack of 22:6n-3 synthesis observed in PBMCs. In combination
with the data in Chapter 3, the findings from this chapter suggest that Con A mediated upregulation of n-3 PUFA synthesis is controlled at the transcriptional level. Elucidation of the molecular mechanisms controlling the transcription of these genes would be important for understanding the regulation of n-3 PUFA synthesis in activated PBMCs.
Chapter 5: Do changes in \textit{FADS2} and \textit{FADS1} gene expression levels involve a change in the DNA methylation status of their promoter regions?
5.1 Introduction

In the previous chapters, it was shown that activation of PBMCs by Con A resulted in significantly higher synthesis of n-3 PUFAs derived from 18:3n-3 and this was accompanied by significant increases in the mRNA expression levels of FADS2, FADS1 and ELOVL5. This suggested transcriptional control of n-3 PUFA synthesis in PBMCs. As discussed in section 1.11, epigenetic modifications have been shown to be related to changes in the mRNA expression of specific genes.

In particular, DNA methylation of cytosines in CpG dinucleotides is an important mechanism by which gene expression can be regulated. DNA methyltransferase (DNMT) enzymes are responsible for establishment and maintenance of DNA methylation; DNMT3a and DNMT3b induce DNA methylation marks, which are maintained through cell division by DNMT1. In general, low methylation of promoter regions is associated with actively transcribed genes, whereas a high methylation is associated with transcriptional silencing (292, 293). DNA methylation can repress transcription directly by interfering with transcription factor binding and indirectly through recruitment of methyl-CpG binding proteins that, in turn, recruit corepressors that modify chromatin (242, 294). Although DNA methylation patterns are primarily established during development and persist throughout the life course, some DNA methylation marks appear to display plasticity in response to environmental changes (251).

Several studies in rodents have shown that increased DNA methylation of the Fads2 promoter, in response to dietary factors, is accompanied by decreased mRNA expression. In a study in mice, postnatal 18:3n-3 supplementation increased average Fads2 methylation in maternal and offspring livers and for maternal livers the level of promoter methylation correlated negatively with Fads2 mRNA expression (259). In rats, changes to the amount and type of dietary fat fed in pregnancy increased methylation at specific CpG loci in the Fads2 promoter and decreased mRNA expression in the liver of adult offspring (213). This was accompanied by lower proportions of 22:6n-3 and 20:4n-6 in membrane phospholipids and the study also showed that hepatic Fads2 mRNA expression correlated negatively with methylation of four CpG dinucleotides in the Fads2 promoter (213). In another study in rats, feeding dams increased dietary fat resulted in increased methylation of specific CpG loci in the Fads2 promoter, which was accompanied by a decrease in mRNA expression in aortae of adult offspring (260). Mutation of a specific CpG dinucleotide that was hypermethylated confirmed its role in regulating Fads2 transcription. This CpG dinucleotide was located in a putative oestrogen receptor response element and oestrogen treatment induced a significant dose-related increase in activity of the wild type FADS2 promoter but had no effect on the mutated promoter (260). Hypermethylation of the Fads2 promoter and lower mRNA expression and delta-6 desaturase activity have also been observed in livers of mice with hyperhomocysteinemia (HHcy) compared with wild type mice (295). Together these studies provide
evidence that regulation of FADS2 transcription by DNA methylation may contribute to the regulation of PUFA synthesis in rodents.

Plasticity in the methylation status of CpG dinucleotides in the FADS2 proximal promoter has also been demonstrated in human PBMCs isolated from patients with renal disease (261). The methylation status of specific CpG dinucleotides located in a region extending approximately 1000 bp from the FADS2 transcription start site (TSS) were altered in response to dietary supplementation with n-3 PUFAs or olive oil when compared to baseline. Furthermore, the methylation levels of four CpG dinucleotides were associated negatively with the level of their transcripts. This suggests that the level of methylation in the region directly upstream of the FADS2 could be important in transcriptional regulation in human PBMCs.

The close proximity of FADS2 and FADS1 and their divergent transcription configuration suggests their transcription may be coordinated by regulatory factors in the genomic region between the genes. The DNA methylation status of a specific CpG dinucleotide (cg27386326) situated in this intergenic region has been shown to be associated with delta-6 and delta-5 desaturase activities in human liver samples (273). Bioinformatics analysis revealed that this CpG dinucleotide is positioned in a putative enhancer (273). Enhancers are distinct genomic regions that generally act from a distance to upregulate the transcription of a target gene from its TSS. Active enhancers are bound by activating transcription factors and are brought into proximity of their respective target promoters by looping (296). Therefore, CpG dinucleotides in the putative enhancer region located between FADS1 and FADS2 could be important in regulating their transcription in addition to loci in the region directly upstream of the TSS.

DNA methylation changes are known to be important in regulating the expression of genes involved in T-lymphocyte activation. For example, when naïve CD4+ T-lymphocytes are stimulated by antigen they differentiate into one of several lineages of Th-lymphocytes including Th1- and Th2-lymphocytes, which are defined by their pattern of cytokine production (135). This involves reciprocal activation and silencing of specific cytokine genes, which is fundamental to the differentiation process. Several studies have indicated the importance of promoter DNA methylation in regulating transcription of cytokine genes. For example, during Th1 lineage commitment the promoter region of the IFN-γ gene undergoes progressive demethylation and activation while the IL-4 gene remains hypermethylated and silenced. Conversely, Th2 lineage commitment involves progressive hypomethylation and activation of IL-4 where IFN-γ gene remains hypermethylated and silenced (297-301). However, it has been shown that demethylation is not required for primary transcription of the IL-4 gene but instead is strongly associated with high level IL-4 transcription (301). Demethylation is believed to be a passive process whereby during cell
division the newly replicated DNA strand does not become methylated (229). However, demethylation and enhanced transcription of another cytokine (IL-2) gene following activation occurs before commencement of cell division suggesting an active mechanism for demethylation (302, 303). The DNA methylation changes discussed are stable and heritable providing a cellular memory enabling reiteration of cytokine gene expression upon re-encounter with antigen in a manner that is independent of the cell cycle, facilitating rapid cytokine production in a secondary immune response (302). Finally, in addition to DNA methylation changes, H3 and H4 histone acetylation has been shown to increase in the IFN-γ and IL-4 loci following TH1 and TH2 differentiation, respectively, providing accessibility for transcription (263, 264).

In summary, DNA methylation is known to be important in the regulation of gene expression in activated T-lymphocytes and in studies in rodents and human PBMCs, FADS2 transcription has been shown to be related to the DNA methylation status of its promoter region. However, it is not known whether the increase in FADS2, FADS1 and ELOVL5 mRNA expression measured in activated PBMCs involves altered DNA methylation.

Aims

To investigate whether in vitro activation of PBMCs induces altered DNA methylation of specific CpG dinucleotides located in the FADS2 and FADS1 proximal promoters and intergenic region and to characterise the effect of cell culture on the methylation status of these CpG dinucleotides. This chapter also aims to investigate whether DNMT mRNA expression is altered in response to Con A activation.

Hypotheses

- The methylation levels of specific CpGs measured in the FADS2 and FADS1 promoter regions and intergenic region are significantly lower in response to activation.
- The mRNA expression levels of DNMT1, DNMT3a and DNMT3b are significantly lower in response to activation.

To address these aims and hypothesis, percentage DNA methylation was compared in PBMCs cultured in the presence and absence of the T-lymphocyte mitogen Con A, using pyrosequencing assays designed to measure CpG dinucleotides located in the FADS2 and FADS1 promoters and intergenic region. The DNA methylation levels of these CpG dinucleotides were also measured in freshly isolated PBMCs in order to characterise the effect of cell culture on methylation status. Secondly, DNMT1, DNMT3a and DNMT3b mRNA expression was compared in unstimulated and Con A stimulated PBMCs. Finally, to follow on from the findings from the DNA methylation comparisons, a technique called 5’RACE was carried out to characterise the FADS2 TSS and in silico
analysis was performed to predict transcription factor binding sites in the regions measured in the pyrosequencing assays.
5.2 Methods

5.2.1 Experimental Design

All PBMC samples were obtained from volunteers participating in the EPUFA study described in section 2.2. PBMCs were isolated and cultured in the presence and absence of Con A using the experimental design described in Section 3.2 and summarised in Figure 3.1, Chapter 3. The subject characteristics and PBMC phenotypes are given in Table 3.1 and Table 3.2, respectively. Following culture, PBMC pellets were collected and frozen (section 2.6.1) ready for subsequent DNA extraction. In addition, PBMC pellets from 10 of the volunteers (8 male and 2 female) were also collected and frozen immediately after isolation without undergoing cell culture (section 2.4).

5.2.2 DNA extraction and pyrosequencing analysis

DNA was extracted from PBMC pellets (section 2.14.3) and sodium bisulphite pyrosequencing (section 2.16) was used to measure the DNA methylation status of CpG dinucleotides in the regions adjacent to the human FADS2 and FADS1 transcription start sites. The CpG dinucleotides measured were in a region between -18 and -1661 from the TSS for FADS2 (Figure 2.15) and +64 and -713 bp from the TSS for FADS1 (Figure 2.16). DNA methylation levels of nine CpG dinucleotides, located in the intergenic region 7397 bp and 3202 bp from FADS2 and FADS1, respectively were also measured (Figure 2.17).

5.2.3 DNMT1, DNMT3a and DNMT3b mRNA expression

DNMT mRNA expression analysis was carried out by real-time RT-PCR using the procedure described in section 2.15.

5.2.4 5’ RACE

5’ RACE (section 2.17) was carried out to determine the sequence at the 5’ end of the FADS2 transcript and therefore characterise the TSS in cultured PBMCs treated with and without Con A. Purchased cryopreserved PBMCs were revived and cultured (section 2.8) using the experimental design and conditions described in section 3.2. Two different RACE procedures were performed on RNA extracted (section 2.14.2) from PBMC pellets: RNA ligase mediated (RLM) 5’ RACE, which only amplifies cDNA from full length capped mRNA (section 2.17.1) and SMARTer® 5’ RACE (section 2.17.2). Cloned 5’RLM-RACE (2.17.4.2) and SMARTer® 5’ RACE products (2.17.4.4) were sent for sequencing as described in section 2.17.4.7.
5.3 Results

5.3.1 FADS2 promoter methylation status

Overall, the level of FADS2 methylation increased with increasing distance from the TSS (Figure 5.1, graph A). The region displayed a relatively highly methylated domain located distal to the TSS followed by a sharp decrease by approximately a third at -1119. There was a transitionary region with generally increasing methylation between -1112 and -975, an intermediate region between -914 and -667 and then a very lowly methylated region approaching the TSS. Mean differences in methylation levels between untreated (unstimulated) and Con A treated (stimulated) PBMCs are displayed in Figure 5.1, graph B. Con A stimulation significantly increased the methylation status of 12 CpG dinucleotides located at -1278, -1156, -1112, -1101, -1071, -1067, -1013, -980, -975, -871, -686 and -667 ranging between a difference of 1.1 to 11.4 percentage points (P < 0.001 for all). When male and female groups were analysed separately (data not shown) the methylation status of CpG -1112 was significantly increased in both males and females. Females also showed a significant increase in methylation at -975, and -871 and males at -1156, -1101 and -1067 (P < 0.001 for all).

The methylation profile of PBMCs that had not undergone cell culture (pre-culture) was similar to the profile of unstimulated cultured PBMCs (post-culture) as shown in Figure 5.2 (graph, A). Mean differences in methylation levels between pre-culture and unstimulated PBMCs are displayed in Figure 5.2, graph B). For most of the CpG dinucleotides measured, methylation was increased in cultured compared with pre-culture PBMCs, however, this was only statistically significant for -1661 (P < 0.001).
Figure 5.1. The effect of PBMC activation on the methylation of individual CpG loci in the \textit{FADS2} promoter.

Values are mean ± SEM, \( n = 25 \) to 33. Locations of CpG dinucleotides are relative to the TSS. \( \textbf{(A)} \) Mean methylation in unstimulated (0 \( \mu \)g/ml Con A) and Con A stimulated (5 \( \mu \)g/ml Con A) PBMCs. \( \textbf{(B)} \) Mean difference in methylation (stimulated minus unstimulated). A positive value represents an increase in methylation in Con A stimulated PBMCs and a negative value represents a decrease. *Statistically significant mean difference in methylation determined by paired-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
Figure 5.2. The effect of PBMC culture on the methylation of individual CpG loci in the FADS2 promoter. Values are mean ± SEM, n = 7 to 10. Locations of CpG dinucleotides are relative to the TSS. (A) Mean methylation in uncultured (pre-culture) and unstimulated cultured PBMCs (post-culture). (B) Mean difference in methylation (post-culture minus pre-culture). A positive value represents an increase in methylation in cultured PBMCs and a negative value represents a decrease. *Statistically significant mean difference in methylation determined by paired-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
5.4  *FADS1* promoter methylation status

The level of methylation of *FADS1* tended to be inversely related to the distance from the TSS but the transition from low to high methylation was sharper than for *FADS2* (Figure 5.3, graph A). There was a highly methylated region (> 60%) distal to the TTS, which then halved to approximately 30% at CpG -262, followed by a downstream region of consistently low methylation (< 5%). Mean differences in methylation levels between unstimulated and stimulated PBMCs (stimulated minus unstimulated) are displayed in Figure 5.3 (graph, B). Con A stimulation significantly increased the methylation status of one CpG dinucleotide located at -262 bp from the TSS (P < 0.001). When male and female groups were analysed separately (data not shown) the methylation status of -430, -262, -94 and -88 were significantly increased in females but no CpG dinucleotides reached the adjusted level for statistical significance (P < 0.001) in males.

The pattern of methylation in pre-culture PBMCs was consistent with that measured in post-culture samples (Figure 5.4, graph A). Methylation increased at all CpG dinucleotides measured in post-culture compared with pre-culture samples (Figure 5.4, graph B) but did not reach the adjusted level for statistical significance.
Figure 5.3. The effect of PBMC activation on the methylation of individual CpG loci in the FADS1 promoter.

Values are mean ± SEM, n = 28 to 33. Locations of CpG dinucleotides are relative to the TSS. (A) Mean methylation in unstimulated (0 µg/ml Con A) and Con A stimulated (5 µg/ml Con A) PBMCs. (B) Mean difference in methylation (stimulated minus unstimulated). A positive value represents an increase in methylation in Con A stimulated PBMCs and a negative value represents a decrease.*Statistically significant mean difference in methylation determined by paired-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
Figure 5.4. The effect of PBMC culture on the methylation of individual CpG loci in the *FADS1* promoter.

Values are mean ± SEM, n = 8 to 10. Locations of CpG dinucleotides are relative to the TSS. (A) Mean methylation in uncultured (pre-culture) and unstimulated cultured PBMCs (post-culture). (B) Mean difference in methylation (post-culture minus pre-culture). A positive value represents an increase in methylation in cultured PBMCs and a negative value represents a decrease. Mean differences in methylation were analysed by paired-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
5.4.1 *FADS2/FADS1* intergenic region methylation status

All CpG dinucleotides measured in the putative enhancer in the *FADS2/FADS1* intergenic region were highly (> 75%) methylated (Figure 5.5, graph A). Mean differences in methylation levels between unstimulated and stimulated PBMCs are displayed in Figure 5.5 (graph, B). Stimulation of PBMCs with Con A significantly decreased the level of methylation compared to untreated cells at CpG loci 0 and +113 (P < 0.001), although the differences were small at 3.5 and 2.1 percentage points, respectively. There were no significant differences when male and female were analysed separately (data not shown).

The methylation profile of uncultured PBMCs was similar to that of cultured samples (Figure 5.6, graph A). There were no significant differences in methylation status between pre-culture and post-culture PBMC samples (Figure 5.6, graph B).
Figure 5.5. The effect of PBMC activation on the methylation of individual CpG loci in the \textit{FADS2/FADS1} intergenic region.

Values are mean ± SEM, n = 28 to 32. Locations of CpG dinucleotides are relative to cg27386326. (A) Mean methylation in unstimulated (0 µg/ml Con A) and Con A stimulated (5 µg/ml Con A) PBMCs. (B) Mean difference in methylation (stimulated minus unstimulated). A positive value represents an increase in methylation in Con A stimulated PBMCs and a negative value represents a decrease. *Statistically significant mean difference in methylation determined by paired-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
Figure 5.6. The effect of PBMC culture on the methylation of individual CpG loci in the FADS2/FADS1 intergenic region.

Values are mean ± SEM, n = 10. Locations of CpG dinucleotides are relative to cg27386326. (A) Mean methylation in uncultured (pre-culture) and unstimulated cultured PBMCs (post-culture). (B) Mean difference in methylation (post-culture minus pre-culture). A positive value represents an increase in methylation in cultured PBMCs and a negative value represents a decrease. Mean differences in methylation were analysed by paired-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
5.4.2 *DNMT1, DNMT3a and DNMT3b* mRNA expression

Due to limited sample amount, preliminary analysis of DNMT expression was performed for seven paired PBMC samples. There were significant increases in *DNMT1* and *DNMT3a* expression ($P < 0.05$) in Con A stimulated compared with unstimulated PBMCs (Figure 5.7). *DNMT3b* expression also increased but did not reach statistical significance.

![Graphs showing mRNA expression of DNMT1, DNMT3a, and DNMT3b](Image)

**Figure 5.7. mRNA expression of *DNMT1, DNMT3a* and *DNMT3b*.**

**Left:** mRNA expression normalised to *EIF4A2, 18S, SDHA* and *RPL13A* for unstimulated (0 µg/ml Con A) and stimulated (5 µg/ml Con A) PBMCs. **Right:** Difference scores (stimulated minus unstimulated) for mRNA expression showing median and IQR. A positive value represents an increase in mRNA expression in Con A stimulated PBMCs and a negative value represents a decrease. Statistical analysis was by Wilcoxon signed-rank test for DNMT1 ($n = 7$), DNMT3a ($n = 7$) and DNMT3b ($n = 6$).
5.4.3 Characterisation of the FADS2 TSS in unstimulated and stimulated PBMCs by 5’ RACE

Pyrosequencing analysis suggested that there might be an alternative start site for FADS2 transcription in stimulated cells compared to unstimulated cells given the wide region of increased methylation observed. Therefore, a technique called 5’RACE was carried out to characterise the FADS2 TSS and to determine if there are different transcripts in unstimulated and Con A stimulated PBMCs.

5.4.3.1 5’ RLM-RACE cDNA control PCR

First, 5’ RACE was carried out using the 5’RLM-RACE kit. In 5’RLM-RACE, ligation of an adapter sequence to the 5’ end of mature mRNA molecules followed by cDNA conversion enables amplification of the 5’ end of the transcript by PCR. To verify the presence of the FADS2 transcript following the 5’ RLM-RACE cDNA conversion step, a control PCR was carried out using the Gene Specific 5’ Control Primer and the Gene Specific Inner Primer (Table 2.6). These primers were designed to bind within the FADS2 transcript so the RT and PCR reactions could be evaluated independently of the CIP, TAP and ligation reactions. This was also carried out for a positive control sample prepared from mouse thymus RNA using primers for the Cxcr4 gene provided with the 5’ RLM-RACE kit. The amplified products were of the expected size for the positive control sample for both the 5’ RACE outer and inner primers (Figure 5.8, B). The FADS2 primers also amplified the expected size product in Con A stimulated samples (Figure 5.8, A), but no PCR product could be amplified in unstimulated samples, perhaps because the level of this transcript was too low. It was decided to proceed with 5’ RACE nested PCR just in stimulated samples to determine whether the TSS matched the published TSS (273).
5.4.3.2 Nested PCR for 5' RLM-RACE

Nested PCR was performed to amplify the 5’ end of the FADS2 and positive control (Cxcr4) transcripts. The products obtained after the inner PCR are shown in Figure 5.9. The positive control produced a band of the expected size (301 bp) indicating that the 5’ RLM RACE and PCR had been successful and the minus TAP control indicated that the product was specific to de-capped, full length mRNA. A smear of non-specific product was visible in the minus TAP control but the 5’ RLM RACE handbook states that this is not a concern. A larger second band was visible in the positive control, which according to the 5’RLM RACE handbook happens when the PCR has a very high yield. The FADS2 inner PCR produced a product of approximately 500 bp. The minus TAP control, which should have no visible PCR product, produced a product of approximately 300 bp suggesting that the FADS2 5’RACE was not specific to full length mRNA. However, since the positive control was successful and for FADS2 the minus TAP control produced a 200 bp smaller product than the TAP treated sample, it was decided to sequence both FADS2 products.
Figure 5.9. FADS2 5’ RLM RACE nested PCR.
Agarose gel showing inner products from nested PCR on Con A stimulated PBMC (+C) and also mouse thymus positive control (+Ctrl) 5’ RACE samples including minus TAP (-TAP) control samples for each and a no template control (NTC).

5.4.3.3 Cloning and sequence analysis of FADS2 5’ RLM-RACE products

To identify the sequence of the FADS2 5’ RLM RACE transcripts, PCR products were inserted into the pCR™4-TOPO® plasmid vector and cloned. Restriction digest confirmed the presence of the expected sized inserts (approximately 500 bp for the TAP treated and 300 bp for the minus TAP control RACE products) in the plasmid vector (Figure 5.10) before three clones for each product were sequenced in both directions. The Basic Local Alignment Search Tool (BLAST) confirmed the products matched the FADS2 sequence. Sequenced RACE products contained the expected 36 bp sequence of the 5’ RACE adapter enabling orientation of the 5’ end of the detected FADS2 transcript. Both sequenced RACE products began at exactly the same location, corresponding to the fourth exon of FADS2 variant 1 (RefSeq: NM_004265.3; Ensembl transcript ID: ENST00000278840.8) but in the reverse complement orientation with the sequence proceeding to the middle of the third exon of FADS2 variant 1 (Figure 5.11). In the TAP treated RACE product the sequence then joined the sequence corresponding to the 5’UTR in the first exon of FADS2 variant 1 (this time in the same orientation as the published sequence), only 2 bp (or 6 bp since AGGG corresponds to both exon 1 and the reverse complement of exon 3) upstream of the reported TSS. For the minus TAP control RACE product the junction was 204 bp (or 208 bp as interestingly again AGGG could have come from either exon) downstream therefore explaining the 200 bp smaller PCR
product obtained. Both sequences were then analysed up to exon 1, which contained the gene specific inner 5’RACE PCR primer. The complex nature of this arrangement (for it to be real it would have been transcribed from both DNA strands and joined into a single mRNA) and the failed minus TAP control (no PCR product should have been obtained) suggested that the sequence was probably a 5’RACE artefact. Furthermore, the product could not be verified by PCR amplification when tested on control PBMC cDNA (data not shown). However, it could be argued that the level of the transcript, if it were real, was too low to amplify. Interestingly, a FADS2 product starting with the reverse complement of exon 4 (at exactly same nucleotide) had been previously detected in the laboratory by another researcher (Dr Rebecca Clarke-Harris) when 5’ RLM RACE had been performed on RNA from HepG2 cells but the sequence downstream of the reverse complement of exon 4 differed to the sequence obtained in PBMCs. This suggests that the FADS2 5’RACE product detected is a consistent artefact associated with the 5’RLM-RACE kit and therefore it was decided to repeat FADS2 5’RACE using a different kit.

![Figure 5.10. Digests of 5’ RLM RACE PCR – pCR4-TOPO clones with EcoRI and NotI.](image)

Three Con A treated PBMC (+C) and minus TAP control (+C-TAP) FADS2 RACE product pCR4-TOPO clones (C1-C3) were digested with EcoRI to confirm presence of appropriate sized inserts in the plasmid vector (+C ~500 bp and +C-TAP ~300 bp) or NotI to linearise the plasmid. An undigested (uncut) control of one clone is also shown.
Figure 5.11. Sequences of FADS2 5′ RLM RACE products.
Sequence corresponding to the reverse complement of exon 4 (blue), the reverse complement of exon 3 (red) and exon 1 (black) of FADS2 variant 1 are shown in differing colours with sequence corresponding to the 5′UTR underlined. Dotted underline indicates sequence corresponding to both exon 4 and exon 1 of the main FADS2 variant. The reported FADS2 TSS is highlighted blue and the inner gene specific RACE primer is highlighted yellow. (A) FADS2 5′ RLM-RACE product (B) FADS2 –TAP control 5′ RLM-RACE product.

5.4.3.4 5′ SMARTer RACE cDNA control PCR

FADS2 5′ RACE was repeated using a SMARTer® RACE kit. This kit does not require adaptor ligation and uses suppression and step-out PCR techniques to increase specificity. This time, FADS2 5′ RACE was also carried out on RNA extracted from the Jurkat T-lymphocyte leukaemic cell line, in addition to unstimulated and stimulated PBMCs since FADS2 mRNA expression and DNA methylation status was also analysed in Jurkat cells (see Chapter 6). To verify the presence of the FADS2 transcript following the SMARTer® 5′RACE cDNA conversion step, a control PCR was carried out using the same Gene Specific 5′ Control Primer and the Gene Specific Inner Primer (Table 2.6) used to verify the presence of FADS2 transcript in the 5′ RLM-RACE procedure. These primers were designed to bind within the FADS2 transcript and amplified the expected size product (220 bp) for unstimulated and Con A stimulated PBMCs samples and Jurkat cells (Figure 5.12).
intensity of the bands reflects the relative abundance of transcripts measured using real-time RT-PCR in Chapter 3 (Con A stimulated PBMC > unstimulated PBMC).

Figure 5.12. SMARTer® 5’ RACE control PCR to verify presence of FADS2 transcript. Agarose gel showing duplicate FADS2 PCR products (expected size 220 bp) for Con A stimulated (+C) and unstimulated (-C) PBMC and Jurkat (JK) samples and no template control (ntc) reactions.

5.4.3.5 SMARTer® 5’ RACE PCR reactions

Touchdown PCR was performed in order to amplify the 5’ end of the FADS2 transcripts using the gene specific primer designed for the SMARTer® 5’ RACE protocol. RACE ready cDNA was also prepared from mouse total heart RNA provided with the kit and a control PCR was carried out using the supplied control 5’ RACE primer specific for the mouse transferrin receptor gene. The PCR products obtained are shown in Figure 5.13. The positive control produced a band of the expected size (2100 bp) indicating that the cDNA conversion and PCR had been successful. The FADS2 gene specific primer generated a main PCR product of approximately 1000 bp for unstimulated and Con A stimulated PBMC and Jurkat samples.
Figure 5.13. FADS2 SMARTer® 5’RACE PCR.
Agarose gel showing FADS2 5’RACE PCR products for unstimulated PBMC (-C), Con A stimulated PBMC (+C) and Jurkat (JK) samples amplified using the FADS2 gene specific primer (GSP) and a positive control (+Ctrl) sample. No template control (ntc) reactions were ran for the FADS2 GSP and positive control.

5.4.3.6 Cloning and sequence analysis of FADS2 SMARTer® 5’RACE products

To identify the sequence of the FADS2 SMARTer® 5’RACE transcripts, PCR products were inserted into the pRACE plasmid vector provided with the kit and cloned. Restriction digest with EcoRI and HindIII was carried out to confirm the presence of the expected sized inserts (Figure 5.14). Products amplified with the Gene Specific Primer contained an EcoRI recognition site within the insert and consequently digestion produced two bands of approximately 400 bp and 600 bp (the sum of which matched the expected size of the insert). Thirteen clones for each product were sequenced (except for Jurkat samples where eleven clones were sequenced) in both directions. Nucleotide BLAST analysis confirmed the products matched the sequence for FADS2 variant 1 (RefSeq: NM_004265.3; Ensembl transcript ID: ENST00000278840.8). The 5’ ends of the RACE products obtained from unstimulated and Con A stimulated PBMCs and Jurkat samples are shown in Figure 5.15. All sequences proceeded to the location of the GSP in exon 5. Some variation in the start sites of 5’ RACE products from unstimulated and Con A stimulated PBMC samples and Jurkat cells was evident. However, there was also variation between individual clones for each of these samples. Overall, the results indicate that there is no distinct difference in the TSS between unstimulated and Con A stimulated PBMC samples and Jurkat cells.
Figure 5.14. Digests of SMARTer® 5’RACE PCR – pRACE clones with EcoRI and HindIII.

Gel shows example digest for three Con A stimulated PBMC (PBMC + Con A) FADS2 RACE product pRACE clones (C1 to C3). Plasmids were digested with EcoRI and HindIII to confirm presence of the appropriate sized insert of ~1000 bp (image shows bands of ~400 bp and ~600 bp due to presence of an EcoRI recognition site within the insert) in the plasmid vector. Linearised (lin) and undigested (uncut) plasmids are also shown.
Figure 5.15. 5’ sequence of FADS2 SMARTer® 5’RACE products.
The first nucleotide of each sequenced RACE insert clone is highlighted grey with the number above indicating the number of clones corresponding to that particular start site for unstimulated PBMC (highlighted yellow), Con A stimulated PBMC (highlighted green) and Jurkat cells (highlighted pink). Sequence corresponding to exon 1 of FADS2 variant 1 is shown in black with the 5’UTR underlined and sequence corresponding to exon 2 is displayed in red. All sequences proceeded to the position of the Gene Specific Primer (GSP) located in exon 5 (sequence not shown). The reported FADS2 TSS is highlighted in blue and the translation start codon in orange.

5.4.3.7 Analysis of putative transcription factor binding sites

To determine which transcription factors may be binding in the regions that were found to be differentially methylated between unstimulated and Con A stimulated PBMCs, sequences were analysed for putative transcription factor binding consensus sequences using MatInspector software (http://www.genomatix.de/cgi-bin//matinspector). A number of transcription factors were predicted to bind in the differentially methylated regions in the FADS2 and FADS1 promoters and FADS2/FADS1 putative enhancer (Table 5.1).
Table 5.1. Putative transcription factor binding sites in the differentially methylated regions of FADS2, FADS1 and the FADS2/FADS1 intergenic region (continued on page 177)

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<th>Matrix sim.</th>
<th>Reported expression and function in lymphocytes</th>
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<td>+</td>
<td>1.00</td>
<td>0.91</td>
<td>Apoptosis in T-cell acute lymphoblastic leukaemia</td>
<td>(328)</td>
</tr>
<tr>
<td>AHRR</td>
<td>-188/-183/180</td>
<td>tggcgccacgagCTGgcaacagggc</td>
<td>+</td>
<td>1.00</td>
<td>0.93</td>
<td>Lymphoid cell development.</td>
<td>(329)</td>
</tr>
<tr>
<td>MAX</td>
<td>-188/-183/180</td>
<td>cttgcCAGCTgtgcgg</td>
<td>-</td>
<td>1.00</td>
<td>0.91</td>
<td>Proliferation, differentiation and apoptosis</td>
<td>(330)</td>
</tr>
<tr>
<td>HIF-1</td>
<td>-188/-183/180</td>
<td>cggcacgaCTGgcaag</td>
<td>+</td>
<td>1.00</td>
<td>0.92</td>
<td>Cytolytic activity in CD8+ T-lymphocytes</td>
<td>(331)</td>
</tr>
<tr>
<td>CREB</td>
<td>-183/-180</td>
<td>gcggcagACGTgcaacagcag</td>
<td>+</td>
<td>1.00</td>
<td>0.90</td>
<td>Proliferation and survival of mature B-lymphocytes</td>
<td>(332)</td>
</tr>
<tr>
<td><strong>FADS2/FADS1 intergenic region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMYB</td>
<td>0</td>
<td>ctacctcctAACGagcttat</td>
<td>+</td>
<td>1.00</td>
<td>0.90</td>
<td>Proliferation, differentiation and tumorigenesis</td>
<td>(308)</td>
</tr>
<tr>
<td>HIF-1</td>
<td>+113/+117</td>
<td>tgCCACGcccg</td>
<td>-</td>
<td>0.91</td>
<td>0.94</td>
<td>Cytoplastic activity in CD8+ T-lymphocytes</td>
<td>(331)</td>
</tr>
<tr>
<td>KLF7</td>
<td>+113/+117</td>
<td>tggccgGGCGtggcgagtct</td>
<td>+</td>
<td>1.00</td>
<td>0.91</td>
<td>Suggested role in T-lymphocyte development</td>
<td>(333)</td>
</tr>
<tr>
<td>ZKSCAN3</td>
<td>+247</td>
<td>gcacctCCCCacacccggctca</td>
<td>-</td>
<td>1.00</td>
<td>1.00</td>
<td>Highly expressed in chronic lymphocytic leukaemia</td>
<td>(334)</td>
</tr>
<tr>
<td>SREBP1*</td>
<td>+166</td>
<td>(c)ggaTACgtagtc</td>
<td>+</td>
<td>1.00</td>
<td>0.94</td>
<td>Metabolic programming of activated T-lymphocytes</td>
<td>(196)</td>
</tr>
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</table>

Potential binding sites for transcription factors identified using MatInspector core/vertebrate transcription factor database. The sequence containing and surrounding the differentially methylated CpG dinucleotides was analysed. Core sim. (core similarities) refers to base pair matching for the core consensus sequence (capitalised) while Matrix sim. (matrix similarities) refers to overall matching (both are scored out of 1.00). Results are for sequences covering CpGs and with Core sim. and Matrix. Sim ≥ 0.9. *The CpG is outside of the SREBP1 binding sequence by 1 bp (shown in brackets) but was included in results since SREBP1 has been reported to regulate FADS2.
5.5 Discussion

The findings presented in this chapter showed that activation of PBMCs with Con A induced altered methylation of specific CpG dinucleotides in the 5’ regulatory regions and in an intergenic region of two genes (FADS2 and FADS1) that encode the key desaturase enzymes in the PUFA biosynthesis pathway.

The promoter region of FADS2 was characterised by a decline in the level of methylation of individual CpG loci towards the TSS. The methylation profile for uncultured and cultured PBMCs (with and without Con A stimulation) was in agreement with a previously published profile of FADS2 methylation in uncultured PBMCs from patients with renal disease (261). FADS1 promoter methylation also decreased towards the TSS but the transition was sharper than observed in FADS2 with the methylation level stepping down from over 90% to approximately 20% at CpG locus -262. Again, FADS1 methylation was consistent with the previously published profile with the drop in methylation occurring at the same CpG locus reported by Hoile et al., 2014 (261). The methylation level of the majority of CpG dinucleotides measured in FADS2 increased in cultured (unstimulated) compared with freshly isolated PBMCs, by a maximum of 8 percentage points. However, the difference only reached statistical significance for the CpG dinucleotide located furthest from the TSS. Cell culture increased methylation in all of the CpG loci measured in FADS1 but none were significantly different and the greatest difference was only 2 percentage points. The sample size was lower (n = 10) than used in the unstimulated versus stimulated PBMC comparisons (n = 33) and therefore the results may be underpowered. However, overall these results suggest that cultured PBMCs retain the DNA methylation characteristics of freshly isolated cells, making them a suitable model for analysing DNA methylation changes.

Con A treatment significantly increased FADS2 promoter methylation at 12 CpG dinucleotides in a region between -1278 bp and -667 bp from the TSS with the difference in methylation ranging from 1% to 11%. This finding is contradictory to the general view that DNA methylation is a repressive epigenetic mark, since in Chapter 4 FADS2 mRNA expression was shown to increase in stimulated PBMCs. In FADS1, methylation also increased after stimulation but only at one specific CpG dinucleotide from a level of 25% to 30%. However, fewer CpG dinucleotides were measured in FADS1 compared with FADS2 and for both genes there is the possibility that there were methylation changes in CpG dinucleotides not covered by the assays.

One explanation for the increased methylation coinciding with increased gene expression could be the DNA methylation preventing the binding of an inhibiting factor such as a transcriptional repressor. Transcriptional repressor proteins associate with their target genes either directly through a DNA binding domain or indirectly by interacting with other DNA bound proteins. DNA
methylation interfering with repressor binding has not been widely reported but has been implicated as the mechanism explaining methylation at the promoter region of the FOXA2 gene and elevated expression during endoderm development (238). In silico analysis, using MatInspector software, predicted that a number of transcription factors may bind across the region that was differentially methylated in FADS2 (Table 5.1). Many of these transcription factors are reported to function in lymphocyte differentiation and proliferation (references given in Table 5.1). Interestingly, DEC2 and MNT, predicted to bind across CpG -1112, are well characterised repressors of transcription (310, 335). However, DEC2 has also been shown to directly activate genes in T-lymphocytes (311). Many transcription factors can act as both activators and repressors depending on their co-factors. It is important to note that the sequences in Table 5.1 are only putative binding sites for the transcription factors listed and actual binding and the effect of DNA methylation on binding would need to be validated experimentally, for example using an electrophoretic mobility shift assay (EMSA). Interestingly, two transcription factors (ELK1 and SREBP1) known to regulate the transcription of FADS2 (125, 146) were predicted to bind within the region. The predicted binding site for ELK1 was highly methylated in PBMCS (85%) and the predicted site for SREBP1 showed an intermediate level of methylation (20%) but neither were significantly altered by activation. In FADS1, the transcription factor HOXB9 was predicted to bind over CpG -262 (Table 5.1), which was significantly increased in stimulated cells. HOXB9 is a member of the HOX family of transcription factors, which are major regulators of animal developmental programmes and function as both transcriptional activators and repressors (336).

In some cases, CpG methylation has been demonstrated to be required for binding of transcription factors. For example, methylation of the CpG in the cAMP response element (CRE) sequence (TGACGTCA) has been shown to enhance the binding of C/EBPα, which activates specific genes critical in activation of differentiation in various cell types (237). Furthermore, the binding of the SP1 transcription factor to DNA and activation of transcription has been shown to occur even when the CpG dinucleotide within its binding site is methylated (337). Furthermore, in another study SP1 was shown to bind with higher affinity to methylated residues (235). Therefore, the relationship between DNA methylation and gene expression is complex but preference of transcriptional activators for methylated cytosines could provide another potential explanation for the increase in methylation and gene expression observed for FADS2 and FADS1.

So far, the possible explanations for the concurrence of increased methylation and higher gene expression have not addressed why methylation increased over a wide area in FADS2. The extent of increased methylation in FADS2 following Con A treatment could suggest the use of an alternative promoter for transcription in activated cells. Alternative promoters provide an important mechanism by which gene expression can be regulated, influencing both transcript
diversity and gene expression levels. The use of more than one transcription initiation site can permit gene expression under different cellular conditions and individual promoters can provide tissue specific expression (338). A considerable fraction of genes with methylated proximal promoters display elevated expression and in many such cases transcription is initiated at a distal CpG island that functions as an alternative promoter (339). There are alternative transcription initiation sites for different protein coding splice variants of the FADS2 gene. The NCBI Reference Sequence database (RefSeq) lists three protein coding FADS2 transcripts. The TSS for the FADS2 variants 2 (RefSeq: NM_001281501.1) and 3 (RefSeq: NM_001281502.1) are situated over 11 kb upstream of the classical FADS2 variant 1 (RefSeq: NM_004265.3). However, these variants would not have been detected in the gene expression analysis as the amplified exons were unique to FADS2 variant 1; therefore the increased expression measured in stimulated compared with unstimulated PBMCs related to the classical variant.

5'RACE was carried out in order to determine whether the FADS2 TSS differed in Con A stimulated and unstimulated PBMCs. Initially, 5’RACE was conducted using 5’RLM-RACE, which has the advantage of being specific to mature capped mRNA molecules. No transcript could be detected for unstimulated PBMCs in control experiments possibly because the level of the transcript was too low to detect after the RACE specific cDNA conversion. A FADS2 5’RACE product was obtained for stimulated PBMCs but a product was also obtained for the minus TAP control indicating the result was not specific to mature mRNAs. However, the minus TAP product was 200 bp smaller, which is difficult to explain. The RACE product obtained for stimulated PBMCs exhibited a non-collinear arrangement, which would have required transcription from both DNA strands and subsequent fusion into a single mRNA, if it were real. Considering the minus TAP control product and complex exon arrangement, it was concluded that the observed product was most likely an artefact of the nested PCR. Analysis of the arrangement suggested that it was a PCR-generated chimera, which occurs as the result of recombination between similar sequences during amplification (340, 341). The experiment was repeated using a different 5’RACE procedure in order to avoid problems inherent to the 5’ RLM RACE kit components, since a strikingly similar arrangement had been observed in HepG2 cells as detailed in the results. RACE was repeated using the SMARTer 5’RACE kit and this time the procedure was also carried out for a T-lymphocyte cell line (Jurkat) in addition to PBMCs. This confirmed the product obtained in 5’ RLM RACE to be an artefact. Visualisation of RACE products on an agarose gel revealed a main transcript that did not differ in size between samples. Sequencing of RACE products confirmed the products corresponded to the 5’ end of FADS2 variant 1. Although there was a small degree of variation in the start sites of 5’ RACE products from unstimulated and Con A stimulated PBMC samples and Jurkat cells, there was also variation between individual clones from the same sample and no distinct overall pattern. Therefore, it was
concluded that there is no distinct difference in the TSS for FADS2 expressed in unstimulated and Con A stimulated PBMC samples and Jurkat cells. The observed differences could represent true variation in the FADS2 TSS, in other words the TSS is not well defined. Many genes have multiple transcription initiation sites and do not conform to the TATA box model, named for its conserved DNA sequence, where transcription is initiated from a defined nucleotide (342, 343). These are termed TATA-less promoters and represent more than 80% of mammalian protein-coding genes (344). Analysis of the FADS2 sequence revealed no TATA box close to the TSS, which commonly located 25-35 bp upstream. A limitation of the SMARTer 5’RACE procedure was that, unlike 5’ RLM RACE, it does not guarantee specificity for mature capped mRNA molecules. Therefore, the shorter products may have represented partially degraded mRNAs. To minimise the impact of this a large number of clones were sequence maximise the amount of 5’ sequence obtained.

It has been demonstrated that the inhibition of gene expression by low density promoter methylation can be overcome by an enhancer (345). Furthermore, it has been reported that T-lymphocyte differentiation and lineage commitment is associated with changes in DNA methylation in enhancer regions (346). In this study, there was consistently high methylation (> 77%) of CpG dinucleotides measured in a putative enhancer region between FADS1 and FADS2 in both pre-culture and post-culture PBMCs, which is characteristic of CpG residues outside of CpG islands. The methylation levels of two CpG dinucleotides were significantly lower in Con A stimulated compared with unstimulated PBMCs. This included the CpG (cg27386326) for which the methylation level was shown previously to correlate negatively with delta-6 and delta-5 desaturase activity (273) and an adjacent CpG dinucleotide (113 bp downstream). However, it is not known whether the decrease in methylation of 3.5 and 2.1 percentage points would be sufficient to explain the change in magnitude of gene expression. In silico analysis predicted binding sites for transcription factors in this region (Table 5.1) including SREBP1 (known to activate FADS2 transcription) therefore further supporting this region as a possible enhancer for FADS gene expression. However, again transcription factor binding and the influence methylation on binding would need experimental verification.

As discussed earlier, increases in CpG dinucleotide methylation were also observed in cultured PBMCs compared with PBMCs prior to culture. These increases, were similar in terms of the CpG loci altered in unstimulated compared to stimulated cultured cells but the magnitude of the changes were smaller. This suggests that the culture system may be having a stimulatory effect. Alternatively, it could be merely an effect of the PBMCs being in an alien environment. Another explanation could be that the increased methylation was due to the presence of 18:3n-3 in the culture medium, since, in rodents, 18:3n-3 supplementation has been shown to induce FADS2 promoter methylation (259). However, unstimulated and stimulated cultures both had 18:3n-3...
present in the media and methylation was greater in stimulated cells. It could be that capacity for
de novo methylation was greater in stimulated cells, which is supported by results from preliminary
experiments shown in Figure 5.11 that suggested the expression of the DNMTs was higher in Con
A treated PBMCs. It is thought that de novo methylation in somatic cells is mainly restricted to
methylated genomic regions where it is proposed that DNMT3a and DNMT3b have a role in
restoring methylation at CpG sites missed by DNMT1 (347). However, a previous study has shown
active DNMT3b recruitment to the IL-4 locus in developing T-lymphocytes, indicating that it may be
responsible for de novo methylation of this locus (348). Activation of naïve CD4+ T-lymphocytes has
been shown to increase FADS2 expression through mTOR (mechanistic target of rapamycin)
mediated activation of transcription factor SREBP1 (146). Therefore, one theory could be that FADS
gene expression increased due to activation of specific transcription factors under stimulating
conditions and the presence of 18:3n-3 may have attenuated the increased gene expression by
inducing methylation. The effect of 18:3n-3 on methylation status and gene expression could be
determined by repeating the experiments with and without 18:3n-3 in the culture medium.

Finally, the heterogeneous cell population could also explain the simultaneous increase in
methylation and gene expression in stimulated PBMCs. For example, it may be that the methylation
change occurred in a different cell type to the gene expression change. This represents the main
limitation when working with a mixed cell population due to inability to resolve the source of the
change. Furthermore, a change in the proportion of particular cell types during culture could change
the level of methylation without an active mechanism. In order to answer these questions
experiments would need replicating on purified individual populations.

In summary, stimulation of PBMC cultures with Con A induced increased methylation of CpG
dinucleotides in the FADS2 and FADS1 promoter regions but it is not clear how these changes relate
to the regulation of gene expression. In FADS2, DNA methylation increased over a number of CpG
dinucleotides whereas for FADS1 methylation only significantly increased at one CpG dinucleotide.
These findings contradict the increased expression measured in activated cells. Possible
explanations were discussed, including decreased repressor binding, preferential binding of
transcription factors to methylated CpGs, the heterogeneity of the PBMC population, the presence
of 18:3n-3 in the culture medium and alternative promoter usage in activated cells. The latter was
investigated using 5’RACE and it was concluded that there was no distinct difference in the FADS2
TSS between unstimulated and Con A stimulated PBMCs.
Chapter 6: Is PUFA synthesis dysregulated in a spontaneously dividing leukaemic T-lymphocyte cell line?
6.1 Introduction

The findings from the previous chapters showed that activation of n-3 PUFA synthesis occurs in PBMCs activated by the T-lymphocyte mitogen Con A and this was associated with upregulation of the genes encoding three key enzymes that operate in the pathway. Furthermore, PBMC activation was associated with DNA methylation changes in the FADS2 and FADS1 regulatory regions.

Jurkat cells, a leukaemic cell line of human T-lymphocytes, are able to proliferate in culture without need for activation. Consistent with their effects on proliferating primary T-lymphocytes, high concentrations of n-3 PUFAs are known to reduce Jurkat proliferation when added to the culture medium (349, 350). 22:6n-3 has been demonstrated to be the most potent inhibitor, where 100 µM decreased proliferation by 96% compared with 59% for 20:5n-3 (350). Studies have shown that Jurkat cells take up 18:3n-3 and 18:2n-6 from the culture medium and convert them to longer chain n-3 and n-6 PUFAs, respectively, determined by increased proportions of conversion products after supplementation (148, 351). Furthermore, direct measurement of synthesis using stable isotope tracers demonstrated that Jurkat cells are able to synthesise significant amounts of n-3 and n-6 PUFAs (148). Interestingly, no PUFA synthesis was detected in another leukaemic T-lymphocyte cell line (CEM) and was virtually absent in the B-lymphocyte cell lines tested (148). The reported high activity of the PUFA desaturation and elongation pathway suggests PUFA synthesis may be dysregulated in Jurkat cells.

It is well understood that de novo fatty acid biogenesis from two carbon precursors is dysregulated in cancer cells (352). While most human cells show a preference for exogenous sources of fatty acids, cancerous cells display a shift towards de novo fatty acid biosynthesis regardless of the levels of extracellular lipids (149). Cellular proliferation is common to all cancers and requires a constant supply of fatty acids for synthesis of membranes and signalling molecules (353, 354). Studies have indicated that cancer cells synthesise fatty acids predominately for membrane phospholipids (355, 356). An in vivo study using [14C]glucose demonstrated that 93% of labelled esterified fatty acids in the Ehrlich ascites tumour (EAT) model were derived from de novo synthesis by tumour cells, while newly synthesised fatty acids transported from liver and adipose tissue represented less than 7% of radioactivity measured in the EAT (357). Furthermore, studies investigating the effect of inhibition of fatty acid synthase (FAS) on cancer cell proliferation and survival have demonstrated the dependence of various types of cancer cells on endogenous fatty acid synthesis (356, 358, 359). Although the main products of de novo fatty acid synthesis are saturated fatty acids, increased desaturase activity has also been shown in some cancers. Studies have reported elevated stearoyl-CoA desaturase 1 (SCD1) expression and activity and increased MUFA content in cancerous cells (360, 361). Additionally, loss of SREBP activity has been shown to attenuate cancer cell proliferation.
through disruption of SCD1-mediated desaturation of newly synthesised saturated fatty acids, resulting in an imbalance between saturated and monounsaturated fatty acids (362). Upregulation of delta-6 desaturase has also been demonstrated in various cancerous tissues (363, 364). High levels of 18:2n-6 conversion products (18:3n-6, 20:3n-6 and 20:4n-6) have been reported in breast cancer compared with non-cancerous tissue and product to precursor ratios indicated delta-6 desaturase activity was increased in cancerous tissue (363). The study also reported elevated PGE₂ concentration in cancer tissue, which is a metabolite of 20:4n-6 and is generally considered to promote tumour growth by, for example, supporting processes such as inflammation and angiogenesis (365).

In summary, increased de novo fatty acid synthesis is crucial for cancer cell proliferation and there is evidence that desaturase activity is also elevated in cancerous tissues. High levels of PUFA synthesis previously detected in the leukaemic T-lymphocyte Jurkat cell line suggests dysregulation of pathway activity. However, there has been no direct comparison of PUFA synthesis in Jurkat cells and primary T-lymphocytes. Furthermore, transcriptional control of PUFA synthesis in Jurkat cells has not been investigated.

**Aims**

The aims of this chapter are to determine whether PUFA synthesis is dysregulated in Jurkat cells and to determine whether PUFA synthesis is important for Jurkat cell proliferation.

**Hypotheses**

- n-3 PUFA synthesis is higher in Jurkat cells relative to activated PBMCs.
- The mRNA expression levels of the genes encoding the enzymes that function in the PUFA synthesis pathway are higher in Jurkat cells relative to activated PBMCs.
- DNA methylation levels in the FADS2 and FADS1 promoters and putative enhancer region are lower in Jurkat cells relative to activated PBMCs.
- Inhibition of delta-6 desaturase reduces the proliferation of Jurkat cells.

To address these hypotheses the levels of individual n-3 PUFAs synthesised from [1-¹³C]18:3n-3 and the mRNA expression of FADS2, FADS1, ELOVL5 and ELOVL2 were compared in Jurkat cells and Con A stimulated PBMCs. These measures were also compared with the HepG2 liver cancer cell line. HepG2 cells are a relevant model system for investigating PUFA synthesis as studies using radiolabelled 18:3n-3 and 18:2n-6 have demonstrated full pathway activity (366, 367). Furthermore, HepG2 cells have been shown to express the genes encoding both desaturase (FADS2 and FADS1) and elongase enzymes (ELOVL5 and ELOVL2) (104). The DNA methylation status of individual CpG dinucleotides in the FADS2 and FADS1 promoters and intergenic region were
measured to determine if any gene expression differences were associated with DNA methylation changes. Finally, Jurkat cells were treated with the delta-6 desaturase inhibitor SC-26196 to determine how pathway inhibition affects proliferation.
6.2 Methods

6.2.1 Cell culture

All PBMC samples were obtained from volunteers participating in the EPUFA study described in Section 2.2. The subject characteristics and PBMC phenotypes are given in Table 3.1 and Table 3.2, respectively, in Chapter 3. PBMCs were isolated (section 2.4) and cultured for 48 h in the presence of 5 µg/ml Con A and 20 µM [1-\(^{13}\)C]18:3n-3 diluted 1/10 with unlabelled 18:3n-3 as described in section 2.6. PBMC activation was confirmed by measurement of CD69 positive events by flow cytometry (section 2.7). Jurkat and HepG2 cells were cultured using the conditions described in sections 2.11.2 and 2.11.1, respectively. For the analyses described below Jurkat and HepG2 cells were cultured for 48 h in the presence of 20 µM [1-\(^{13}\)C]18:3n-3 diluted 1/10 with unlabelled 18:3n-3. For comparison of n-3 PUFA synthesis in Jurkat and HepG2 cells cultured with 10 µM or 20 µM of [1-\(^{13}\)C]18:3n-3 diluted 1/10 with unlabelled 18:3n-3, cells were incubated for 72 h.

6.2.2 Measurement of n-3 PUFA synthesis

\(^{13}\)C-label incorporation into specific n-3 PUFAs was measured by a combination of GC and GC-C-IRMS as detailed in section 2.12 with normalisation to total cell protein (section 2.13).

6.2.3 Measurement of mRNA expression

Total RNA was extracted from PBMC, Jurkat and HepG2 cell pellets as described in section 2.14.2. The mRNA expression of FADS2, FADS1, ELOVL5 and ELOVL2 was measured by real-time RT-PCR as detailed in section 2.15.

6.2.4 DNA extraction and pyrosequencing analysis

DNA was extracted from PBMC and Jurkat cell pellets (section 2.14.3) and sodium bisulphite pyrosequencing (section 2.16) was used to measure the DNA methylation status of CpG dinucleotides in the regions adjacent to the human FADS2 (Figure 2.15) and FADS1 (Figure 2.16) TSS and in an intergenic region between FADS2 and FADS1 (Figure 2.17).
6.2.5 The effect of SC-26196 treatment on PUFA synthesis and proliferation in Jurkat cells

Jurkat cells were incubated with 0 nM (0.02 % (v/v) DMSO vehicle control), 50 nM, 100 nM or 200 nM SC26196 and 20 µM [1-13C]18:3n-3 or 20 µM [U-13C]18:2n-6 diluted 1/10 and 1/60 with unlabelled 18:3n-3 and 18:2n-6, respectively, for 48 h. Six replicate cultures were prepared for each treatment. [13C] label incorporation into specific n-3 and n-6 PUFAs was measured by a combination of GC and GC-C-IRMS with normalisation to total cell protein. Analysis of the effect of SC-26196 treatment on Jurkat cell proliferation was kindly carried out by Eduardo Perez-Mojica. Jurkat cells were seeded at 100,000 cells/ml in 50 ml medium and incubated with 0 nM or 200 nM SC-26196 for 144 h. Cells were harvested every 24 h and viable cells were counted using a haemocytometer after staining with 0.4% trypan blue to distinguish dead cells. Ten replicate cultures were prepared for each treatment and time point.
6.3 Results

6.3.1 n-3 PUFA biosynthesis

The levels of $^{13}$C-labelled n-3 PUFAs were measured in stimulated PBMCs and in Jurkat and HepG2 cells in order to compare the activity of the PUFA biosynthesis pathway (Figure 6.1). Jurkat cells displayed stable isotope enrichment throughout the pathway. An enriched peak was also detected for 18:4n-3, but was close to the detection limit and was consequently only quantifiable in five of the ten replicates (data not shown); for these replicates mean $^{13}$C18:4n-3 normalised to total cell protein was 0.35 pmol/µg cell protein. In HepG2 cells and PBMCs, there was no detectable 18:4n-3 peak, and in PBMCs no enrichment was detected in 22:6n-3. Incorporation of 18:3n-3 was significantly higher in PBMCs compared with Jurkat (P < 0.0001) and HepG2 cells (P < 0.01). $^{13}$C20:3n-3 was 2.5 and 2.2 times higher in PBMCs and Jurkat cells, respectively, compared with HepG2 cells, but was not significantly different. Jurkat cells synthesised higher amounts of 20:4n-3, 20:5n-3 and 22:5n-3 than the other cell types (P < 0.0001). In HepG2 cells, the amount of $^{13}$C20:5n-3 was significantly higher (17-fold) than in PBMCs (P < 0.0001). The amount of $^{13}$C22:5n-3 measured in Jurkat cells was 27-fold and 142-fold higher than in HepG2 cells and PBMCs, respectively (P < 0.0001). Stable isotope enrichment was detected in 22:6n-3 in Jurkat cells but the amount of $^{13}$C22:6n-3 measured was negligible compared with the amounts of $^{13}$C20:4n-3, $^{13}$C20:5n-3 and $^{13}$C22:5n-3, which were 62-, 138- and 236-fold higher than $^{13}$C22:6n-3, respectively. Synthesis of $^{13}$C22:6n-3 was also detected in HepG2 cells, but again there was considerably higher synthesis of $^{13}$C20:4n-3 (7.5-fold), $^{13}$C20:5n-3 (28-fold) and $^{13}$C22:5n-3 (5-fold).

Since low amounts of $^{13}$C22:6n-3 were detected in both Jurkat and HepG2 cells, additional analysis of n-3 PUFA synthesis in Jurkat and HepG2 cells was carried out. Cells were treated with 10 µM or 20 µM $^{13}$C18:3n-3 diluted 1/10 with unlabelled 18:3n-3 to determine whether increasing the amount of 18:3n-3 increased 22:6n-3 synthesis. In both cells the amount of $^{13}$C18:3n-3 and $^{13}$C20:5n-3 and $^{13}$C22:5n-3 increased significantly with increasing $^{13}$C18:3n-3 concentration (Figure 6.2). The amount of $^{13}$C22:6n-3 also increased in HepG2 cells but did not change in Jurkat cells. This suggests that 22:6n-3 is not synthesised in Jurkat cells and it is possible that the [13C]-enrichment detected could have been be due to incomplete resolution of 22:6n-3 from the large enriched 22:5n-3 peak (Figure 6.3).
Figure 6.1. Comparison of n-3 PUFA synthesis in PBMC, Jurkat and HepG2 cells.
Values are mean ± SEM for Con A stimulated PBMCs (n = 32), Jurkat cells (n = 9 replicates) and HepG2 cells (n = 6 replicates). Statistical analysis was by one-way ANOVA with Tukey’s post hoc test. Significant differences are indicated by different letters (P < 0.01 for all).
Figure 6.2. The effect of 18:3n-3 concentration on n-3 PUFA synthesis in Jurkat and HepG2 cells. Cells were treated with 10 µM or 20 µM of [1-13C]18:3n-3 diluted 1/10 with unlabelled 18:3n-3. Values are mean ± SEM (n = 6 replicates). Statistical analysis was by independent-samples t-test for each n-3 PUFA. ****P < 0.0001, ***P < 0.001, **P < 0.01.
Figure 6.3. GC-C-IRMS Chromatograms showing n-3 PUFAs.
The chromatograms were obtained from GC-C-IRMS analysis of fatty acid methyl esters from PBMC, Jurkat and HepG2 cultures incubated with $[1^{13}C]18:3n-3$. The peaks corresponding to 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3 are labelled.

6.3.2 FADS2, FADS1, ELOVL5 and ELOVL2 mRNA expression

The mRNA expression levels of the genes encoding the PUFA pathway enzymes were compared between activated PBMCs, Jurkat and HepG2 cells to determine whether their expression reflects the activity of the pathway. The mRNA expression levels of FADS2, FADS1, and ELOVL5 were significantly higher in Jurkat cells relative to PBMCs and HepG2 cells (Figure 6.4). FADS2 showed the greatest fold difference in expression (30-fold) in Jurkat cells compared with PBMCs followed by FADS1 and ELOVL5 that were 25-fold and 9-fold, respectively ($P < 0.0001$ for all). Expression levels of FADS2, FADS1 and ELOVL5 in Jurkat cells were 8-, 2- and 9-fold that in HepG2 cells ($P < 0.0001$ for all), respectively. There were no significant differences in FADS2 and ELOVL5 expression between PBMCs and HepG2 cells but FADS1 was significantly higher (13-fold) in HepG2 cells ($P < 0.0001$). For ELOVL2, only 12 out of 34 PBMC samples had quantifiable levels of expression. ELOVL2 expression was measurable in Jurkat cells but was close to the detection limit, whereas ELOVL2
expression in HepG2 cells was approximately 150- and 900-fold that in Jurkat cells and PBMCs, respectively.

Figure 6.4. Comparison of FADS2, FADS1, ELOVL5 and ELOVL2 mRNA expression in PBMCs, Jurkat and HepG2 cells.
Values are mean ± SEM for Con A stimulated PBMCs (n = 32, 31, 28 and 12, for FADS2, FADS1, ELOVL5 and ELOVL2, respectively) and Jurkat and HepG2 cells (n = 10 replicates for all genes). Statistical analysis was by one-way ANOVA with Tukey’s post hoc test. Significant differences are indicated by different letters (P < 0.0001 for all).

6.3.3 DNA methylation in the FADS2 and FADS1 promoter and intergenic regions

The DNA methylation status of individual CpG loci in the FADS2 and FADS1 promoters and intergenic region were compared between PBMCs and Jurkat cells in order to determine whether the measured gene expression differences were associated with differences in DNA methylation. Jurkat cells showed the same overall pattern of increasing methylation with increasing distance from the FADS2 TSS as seen in PBMCs (Figure 6.5). However, there were significant differences in the methylation levels of individual CpG dinucleotides over a 994 bp region. The methylation level of the most distal CpG locus measured was significantly higher, by 8 percentage points, in Jurkat cells.
compared with PBMCs (P < 0.0001). All CpG dinucleotides between -1655 and -667 bp had significantly lower methylation in Jurkat cells with the difference in methylation ranging from 7 to 49 percentage points. For 17 out of 24 CpG loci in this region, the level of methylation was over 50% lower in Jurkat cells compared to PBMCs.

*FADS1* displayed a highly methylated region distal to the TSS, followed by sharp drop in methylation at CpG locus -262 and then a lowly methylated region proximal to the TSS in both PBMCs and Jurkat cells (Figure 6.6). Again, the methylation level of the most distal (relative to the TSS) CpG locus (-713) measured was significantly higher, by 8 percentage points, in Jurkat cells compared with PBMCs (P < 0.0001). The methylation levels of two CpG dinucleotides, -430 and -262, were significantly lower in Jurkat cells by 6 and 10 percentage points, respectively (both P < 0.0001). There was a significantly (P < 0.0001) lower level of methylation at CpG dinucleotides -204 and -88 in Jurkat cells, however, the methylation levels of these CpG dinucleotides were less than 5%, which has been previously been shown to be the detection limit for pyrosequencing (368).

CpG loci surrounding cg27386326, a CpG locus situated in a putative *FADS2/FADS1* enhancer that has shown previously to be associated with delta-6 desaturase activity (273) were analysed to determine if there were differences between PBMCs and Jurkat cells. The methylation level of the CpG locus -144 was significantly higher by 5 percentage points in Jurkat cells compared to PBMCs, where the methylation level of the CpG locus +209 was 20 percentage points lower in Jurkat cells (Figure 6.7).
Figure 6.5. Methylation of individual CpG loci in the FADS2 promoter in PBMC and Jurkat cells. Values are mean ± SEM for Con A stimulated PBMCs (n = 25 to 33) and Jurkat cells (n = 8 to 10). Locations of CpG dinucleotides are relative to the TSS. *Means that were significantly different determined by independent-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
Figure 6.6. Methylation of individual CpG loci in the FADS1 promoter in PBMC and Jurkat cells.
Values are mean ± SEM for Con A stimulated PBMCs (n = 28 to 33) and Jurkat cells (n = 9 to 10).
Locations of CpG dinucleotides are relative to the TSS. *Means that were significantly different determined by independent-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.

Figure 6.7. Methylation of individual CpG loci in the FADS2/FADS1 intergenic region in PBMC and Jurkat cells.
Values are mean ± SEM for Con A stimulated PBMCs (n = 28 to 33) and Jurkat cells (n = 9 to 10).
Locations of CpG dinucleotides are relative to cg27386326. *Means that were significantly different determined by independent-samples t-test. The P-value for significance was adjusted to P < 0.001 by the Holm-Sidak correction for multiple tests.
6.3.4 The effect of delta-6 desaturase inhibition on Jurkat cell proliferation

Inhibition of delta-6 desaturase activity by SC-26196 was confirmed by measurement of n-3 and n-6 PUFA biosynthesis in Jurkat cells. Since delta-6 desaturase acts on both n-3 and n-6 PUFAs, analysis of both pathways enabled thorough analysis of the effect of SC-26196 on delta-6 desaturase activity. A concentration of 200 nM SC-26196 was recommended by the supplier. SC-26196 has an IC50 of 200 nM and at this concentration has been shown to be a selective and long lasting delta-6 desaturase inhibitor (369, 370). Initial experiments where Jurkat cells were incubated with 0, 50, 100 and 200 nM SC-26196 in the presence of [U-13C]18:2n-6 or [1-13C]18:3n-3 demonstrated a dose response in inhibition of delta-6 desaturase activity and (Figure 6.8). Using n-6 PUFAs, this was determined by measuring the amount of stable isotope labelled substrate ([U-13C]18:2n-6) and its immediate delta-6 desaturase conversion product [U-13C]18:3n-6 at increasing SC-26196 concentrations. For n-3 PUFAs, [1-13C]18:4n-3, the immediate delta-6 desaturase conversion product of [1-13C]18:3n-3 was not detectable so the [1-13C]18:4n-3 elongation product [1-13C]20:4n-3 was measured instead (Figure 6.8). Figure 6.9 shows the effect of 200 nM SC-26196 treatment on all measured PUFAs in the conversion pathway. SC-26196 caused a significant increase in the levels of labelled 18:3n-3 and 18:2n-6 and decreases in the downstream conversion products 20:4n-3, 20:5n-3, 22:5n-3 and 18:3n-6, 20:3n-6, 20:4n-6 and 22:4n-6, respectively. The amounts of labelled 20:3n-3, 20:2n-6 and 22:2n-6 (an elongation product of 20:2n-6), which are synthesised in the alternative delta-8 desaturation pathway, also increased in the presence of SC-26196. The levels of 18:4n-3 and 22:5n-6 were below the detection limit so could not be quantified.

Next, it was determined whether treatment with 200 nM SC-26196 affects the viability and proliferation of Jurkat cells. There was a significant main effect of time on Jurkat cell viability (P < 0.0001) such that the viability of Jurkat cells decreased from 96% to 89% after 144 h of culture in both treated and untreated cells (Figure 6.10, graph A), but there was no significant effect of SC-26196 treatment on viability. There was a statistically significant interaction between SC-26196 treatment and time on Jurkat cell number (P < 0.0001, partial η2 = 0.451). Therefore, simple main effects were analysed. SC-26196 treatment significantly increased the number of Jurkat cells by approximately 1.5 x 10^7 cells after 24 h (P < 0.0001) and significantly decreased the number of cells by approximately 2.5 x 10^7 cells after 144 h (P = 0.451) culture (Figure 6.10, graph B).
Figure 6.8. The effect of SC-26196 concentration on 20:4n-3 and 18:3n-6 synthesis in Jurkat cells. Values are mean ± SEM (n = 6 replicates) for Jurkat cells cultured with increasing concentrations of the delta-6 desaturase inhibitor SC-26196. Statistical analysis was by one-way ANOVA with Dunnett’s post hoc test ***P < 0.001; **P < 0.01; *P < 0.05.
Figure 6.9. The effect of SC-26196 treatment on n-3 and n-6 PUFA synthesis. 
(A) n-3 PUFAs synthesised from [1-\text{13}C]18:3n-3. (B) n-6 PUFAs synthesised from [U-\text{13}C]18:2n-6. Values are mean ± SEM (n = 6 replicates) for Jurkat cells cultured with and without 200 nM of the delta-6 desaturase inhibitor SC-26196. Statistical analysis was by independent-samples t-test. ***P < 0.001; **P < 0.01; *P < 0.05.
Figure 6.10. The effect of SC-26196 treatment on Jurkat cell proliferation.

Jurkat cell viability (A) and number (B) were determined by counting using trypan blue staining and a haemocytometer. Values are mean ± SEM (n = 10 replicates). Statistical analysis was by two-way repeated measures ANOVA. ***P < 0.001; *P < 0.05.
6.4 Discussion

The data presented in this chapter showed that n-3 PUFA synthesis is constitutively active in Jurkat cells. Conversion of 18:3n-3 to 20:4n-3, 20:5n-3 and 22:5n-3 was substantially higher in Jurkat cells compared with PBMCs activated with the T-lymphocyte mitogen Con A. Synthesis was also significantly higher in Jurkat cells compared with the HepG2 cell line, in which full pathway activity has been previously characterised (104, 366, 367). This is in agreement with previous findings that showed the activity of the PUFA synthesis pathway was significantly higher in Jurkat cells relative to other cell lines (148). As discussed in Chapter 3, synthesis of 20:3n-3, a product in the alternative delta-8 desaturation pathway, was prominent in PBMCs. Synthesis of 20:3n-3 was also detected in Jurkat and HepG2 cells indicating that this pathway is also operating in these cell types. However, it is not known whether 20:3n-3 was converted to 20:4n-3 and therefore fed back into the main pathway. The findings from this Chapter demonstrate that n-3 PUFA synthesis is elevated in a leukaemic cell line, which is consistent with reports of increased delta-6 desaturase activity in cancerous cells (363, 364). Possible explanations for increased activity of the PUFA synthesis pathway in Jurkat cells are discussed later in this section.

Stable isotope enrichment in 22:6n-3 indicated synthesis of this fatty acid was active in Jurkat and HepG2 cells. The amounts of synthesised 22:6n-3 were comparable between these two cell types, however, in Jurkat cells synthesis was considerably lower than upstream 18:3n-3 conversion products. The previous publication on n-3 PUFA synthesis in Jurkat cells also reported lower 22:6n-3 synthesis compared with 20:5n-3 and 22:5n-3, measured as 10%, 60% and 20% of total cell radioactivity, respectively (148). In the present study, increasing the concentration of [1-13C]18:3n-3 in the culture medium, as expected, increased the amounts of conversion products [1-13C]20:5n-3 and [1-13C]22:5n-3 in Jurkat and HepG2 cells. The amount of [1-13C]22:6n-3 also increased in HepG2 cells at the higher concentration of precursor but interestingly, remained unchanged in Jurkat cells. One interpretation could be that 22:6n-3 is not synthesised in Jurkat cells and the enrichment detected was due to carryover from the elevated 22:5n-3 synthesis, which produced a large enriched peak beside the 22:6n-3 peak in GC-C-IRMS analysis. This has been previously reported as a limitation of GC-C-IRMS, where isotope measurements for compounds eluting immediately after enriched compounds were significantly affected due to carryover effects (371). A possible solution to this issue would be to run the samples on a different column that reverses the order in which the fatty acids are eluted, for example, by using a low polarity phase, 5% diphenyl/95% dimethyl polysiloxane capillary column (Rtx®-5, Thames Restek). As stated earlier, enrichment in 22:6n-3 has been reported previously in Jurkat cells cultured with radiolabelled 18:3n-3. However, culture supplementation with unlabelled 18:3n-3, in the same study, increased
the proportions of the conversion products 20:4n-3, 20:5n-3 and 22:5n-3 but decreased 22:6n-3 (148). This raises further doubt over the ability of Jurkat cells to synthesise 22:6n-3.

The mRNA expression of FADS2, FADS1 and ELOVL5 were substantially higher in Jurkat cells compared with activated PBMCs and HepG2 cells. This suggests that the high level of n-3 PUFA synthesis observed in Jurkat cells is controlled at the transcriptional level and is in agreement with previous studies that have shown that increased mRNA expression is associated with increased pathway activity (104, 105, 213). ELOVL2 mRNA expression was significantly higher in HepG2 cells relative to both PBMCs (900-fold) and Jurkats (150-fold). ELOVL2 mRNA expression was minimal in PBMCs and Jurkat cells and as discussed in Chapter 4, this may explain the restricted 22:6n-3 synthesis in these cells. This is further supported by the observation that HepG2 cells both synthesised 22:6n-3 and expressed ELOVL2 mRNA, which is consistent with previous reports (104, 366, 367).

Comparison of DNA methylation in activated PBMCs and Jurkat cells revealed substantially lower methylation over a large number of CpG dinucleotides, between 667 bp and 1655 bp upstream of the reported FADS2 TSS, in Jurkat cells. However, for one CpG dinucleotide (-1661) methylation was higher in Jurkat cells. This same region also displayed methylation differences between unstimulated and stimulated PBMCs in this study (Chapter 5) and in response to dietary oil supplementation in another study in PBMCs (261). As shown in Table 5.1 in Chapter 5, this differentially methylated region contains putative binding sites for a number of transcription factors including SREBP1, a known activator of FADS2 transcription (146). As discussed in section 1.11.3, a high level of gene expression is often associated with low promoter methylation (372, 373). Therefore, the findings in this chapter suggest that the lower level of DNA methylation in the FADS2 promoter region might explain the high level of FADS2 transcription observed in Jurkat cells. Consistent with the present findings, previous studies in rat liver and aortae demonstrated that reduced methylation in the Fads2 promoter region was associated with increased Fads2 transcription (213, 260). Furthermore, methylation differences between activated PBMCs and Jurkat cells were also observed in the FADS1 promoter region. The most distal CpG dinucleotide measured relative to the FADS1 TSS displayed significantly higher methylation in Jurkat cells where methylation of four downstream CpG dinucleotides decreased. However, two of these CpG dinucleotides were in a region of very low methylation (<5%). Nonetheless, the methylation changes measured in FADS1 may explain the elevated mRNA expression levels in Jurkat cells. Finally, altered methylation in Jurkat cells relative to activated PBMCs was also measured in a putative enhancer region (273) located in the intergenic region between FADS2 and FADS1. For one CpG dinucleotide, methylation was 5 percentage points higher in Jurkat cells whereas lower methylation by 20 percentage points was measured for a CpG dinucleotide located 37 bp
downstream (relative to the *FADS2* TSS) of a predicted SREBP1 binding site (Table 5.1). These findings suggest that methylation in this predicted enhancer region could be important for the elevated *FADS2* and *FADS1* transcription observed in Jurkat cells relative to activated PBMCs. This complements the findings from a previous study in which methylation of another CpG in this region (cg27386326) was reported to be negatively correlated with delta-6 and delta-5 desaturase activity (273).

The findings from this chapter suggest that n-3 PUFA synthesis is dysregulated in Jurkat cells and reduced methylation could be the mechanism responsible for elevated transcription of the *FADS2* and *FADS1* genes. Aberrant DNA methylation is a common feature of cancerous cells (374). Global loss of DNA methylation, particularly in regions that are normally inactivated, occurs in a wide variety of cancers (256). Hypermethylation in the promoter regions of tumour suppressor genes is one of the most prevalent characteristics of cancer genomes (374). However, promoter hypomethylation and increased gene expression have been reported for specific genes in various cancers including the BCL2 and HOXII genes in leukaemia (375, 376). Therefore, the findings in this chapter are consistent with changes that have been shown for other genes in leukaemic cells.

Since Jurkat cells divide spontaneously in culture without need for activation it was hypothesised that the high level of PUFA synthesis measured in Jurkat cells is important for providing fatty acids to support membrane synthesis in a highly proliferating cell population. However, inhibition of the pathway using the delta-6 desaturase inhibitor SC-26196 only caused a small significant decrease (6%) in the number of cells after 144 h with no significant differences at earlier time points, except after 24 h where the number of cells increased by 16%. Why delta-6 desaturase inhibition caused an increase in cell number after 24 h is not clear. The small decrease in cell number at 144 h might have occurred because exogenous fatty acids in the media were becoming depleted and consequently endogenous PUFA synthesis became important in supporting growth. Repeating the experiment using fatty acid depleted media could answer this question. The measured differences in cell number were small and could represent type I statistical errors. Furthermore, measurement of n-3 and n-6 PUFA synthesis demonstrated that 200 nM SC-26196 did not inhibit PUFA synthesis completely and therefore enough synthesis may have been sustained in the presence of the inhibitor to support cell growth. The results from this experiment are consistent with the results obtained from proliferation analysis in activated PBMCs treated with SC-26196, where only a small reduction in proliferation was observed. Given the high level of PUFA synthesis in Jurkat cells, it is postulated that it serves additional functions to merely supporting cell growth. For example, as discussed in section 1.10.2, n-6 and n-3 PUFAs are precursors to eicosanoids and n-3 PUFAs can also be converted to resolvins. There have been conflicting reports on eicosanoid production in Jurkat cells; some early studies reported that Jurkat cells do not synthesise eicosanoids (168, 377), while
another demonstrated that Jurkat cells are able to convert 20:4n-6 to prostaglandins and thromboxanes (378). More recently, 5-lipoxygenase (5-LOX) activity and leukotriene synthesis have been detected in activated Jurkat cells (379, 380). Furthermore, in a previous study, Jurkat cells incubated with radiolabelled 18:3n-3 and 18:2n-6 released radiolabelled n-3 and to a larger extent, n-6 PUFAs into the culture medium, respectively (148). The main n-3 PUFA released was 20:5n-3 and the main n-6 PUFA was 20:4n-6. The authors highlighted that this reflects the release of 20:4n-6 in normal T-lymphocytes stimulated with PHA, which was shown to be utilised by monocytes for eicosanoid synthesis (286).

Finally, inhibition of delta-6 desaturase activity as expected increased the levels of [1-13C]18:3n-3 and [1-13C]18:2n-6 and decreased [13C]-labelled conversion products in the classical n-3 and n-6 PUFA pathways, respectively. The levels of [13C]20:3n-3 and [13C]20:2n-6, products in the alternative delta-8 desaturation pathway, significantly increased. Furthermore, [13C]22:2n-6, an elongation product of 20:2n-6 was also detected and was significantly increased by SC-26196 treatment. The levels of these products may have risen due to there being more 18:3n-3 and 18:2n-6 available, since conversion to 18:4n-3 and 18:3n-6, respectively, was restricted. This supports the theory that the delta-8 pathway is in competition with the classical pathway and prevails when elongase activity on 18:3n-3 and 18:2n-6 is greater than delta-6 desaturase activity. Therefore, the alternative delta-8 desaturation pathway could provide an important route for PUFA synthesis when delta-6 desaturase activity is restricted. Another explanation could be that delta-6 desaturase catalyses the delta-8 desaturation of 20:3n-3 and 20:2n-6 to form 20:4n-3 and 20:3n-6, respectively. The FADS2 gene product has been shown to be able to delta-8 desaturate 20:3n-3 and 20:2n-6 to 20:4n-3 and 20:3n-6, respectively, in S. cerevisiae transformed with mammalian FADS2 (19). Therefore, if this same activity were present in Jurkat cells inhibition of the delta-6 desaturase enzyme would explain the accumulation of 20:3n-3 and 20:2n-6 due to decreased conversion. Synthesis of 18:4n-3 was not detectable in Jurkat cells, however the n-6 pathway equivalent (18:3n-6) was detected and was significantly decreased by inhibition of delta-6 desaturase confirming that the classical PUFA pathway was operating in Jurkat cells. Synthesis of 20:3n-3 was also detected in PBMCs and HepG2 cells but no 18:4n-3 peak could be detected in either of these cells. A previous study was able to measure 18:4n-3 (1.6% of total radioactivity) and 20:3n-3 (6.8% of total radioactivity) synthesised from radiolabelled 18:3n-3 in HepG2 cells (367). The greater detection of 20:3n-3 in this study does not necessarily mean the delta-8 desaturation pathway is favoured, it could be that conversion of 18:3n-3 to 20:4n-3 happens at much greater rate than conversion of 20:3n-3 and therefore 18:4n-3 does not accumulate so quickly. This is supported by findings from a different study, in a human MCF7 cell system stably transformed with FADS2, where increasing the concentration of 18:3n-3
resulted in greater accumulation of 20:4n-3 compared to 18:4n-3, indicating rapid or coupled elongation of 18:4n-3 (22).

In summary, the findings from this chapter showed substantially higher levels of n-3 PUFA synthesis and FADS2, FADS1 and ELOVL5 mRNA expression in Jurkat cells relative to activated PBMCs. DNA methylation was significantly lower over a large number of CpG dinucleotides in the FADS2 promoter region and lower methylation levels of individual CpG dinucleotides were also detected in the FADS1 promoter and a putative FADS2/FADS1 enhancer region. These findings suggest that PUFA synthesis is dysregulated in Jurkat cells at the level of transcription and lower DNA methylation may be a mechanism through which gene expression is elevated. Partial inhibition of PUFA synthesis only had a minor effect on Jurkat proliferation suggesting that support of new cell membrane synthesis may not be the primary role of PUFA synthesis in Jurkat cells. Nonetheless, the high level of PUFA synthesis measured in Jurkat cells suggest that the activity of the pathway is important for Jurkat cell function. Similar to activated PBMCs, expression of ELOVL2 and synthesis of 22:6n-3 appeared to be restricted in Jurkat cells where both were functioning in HepG2 cells; this suggests that ELOVL2 expression acts as control point for 22:6n-3 synthesis.
Chapter 7: Is the activity of the PUFA biosynthesis pathway influenced by age and gender in activated PBMCs?
7.1 Introduction

As discussed in section 1.7.2 evidence suggests that there are gender differences in the activity of the PUFA biosynthesis pathway. Both 22:6n-3 status and synthesis have been shown to be higher in women compared to men (95, 96, 99, 100, 381, 382). Furthermore, 22:6n-3 status was found to be higher in women taking the oral contraceptive pill and in women receiving hormone replacement therapy (102, 103), which suggests that sex hormones play a role in the regulation of PUFA synthesis. In pregnant rats, positive correlations between Fads2 hepatic mRNA expression and plasma oestradiol and progesterone concentrations have been reported (105). A study in cultured human (HepG2) cells demonstrated that progesterone treatment increased n-3 PUFA synthesis from stable isotope labelled 18:3n-3 and FADS2, FADS1, ELOVL5 and ELOVL2 mRNA expression (104). Furthermore, in the same study progesterone induced increased expression of FADS2 in primary hepatocytes. These findings indicate that gender differences in PUFA synthesis may be mediated through the effects of female hormones on the transcription of genes that encode the enzymes that function in the pathway. In addition to sex hormones, gender differences might also be explained by lower partitioning of 18:3n-3 to β-oxidation in women compared with men therefore affecting the availability of 18:3n-3 for conversion to longer chain n-3 PUFAs (99, 100). Rapid catabolism of 18:3n-3 has been shown to restrict the rate of PUFA synthesis (95, 383).

The effect of aging on n-3 PUFA synthesis is less clear due to fewer studies and conflicting evidence. As detailed in section 1.7.4 comparison of studies in younger and older men indicated that synthesis of 20:5n-3 and 22:5n-3 was lower in older men but there was no difference in 22:6n-3 synthesis (97, 100, 117). Furthermore, studies have reported a decline in delta-6 desaturase activity in rats (120, 121) and humans (118) with age, where another study in humans found no evidence for this (119). Progesterone and oestradiol concentrations decrease following the menopause (384) yet in a previous study no differences in delta-6 desaturation function in pre- and post-menopausal women were found (119). However, the study used the ratio of product to precursor to indirectly estimate desaturation function, which has limited accuracy due to the influence of dietary factors.

In agreement with an earlier study, the findings presented in Chapter 3 demonstrated that activation of n-3 PUFA synthesis occurs upon treatment of PBMCs with a T-lymphocyte mitogen (147). However, no studies have assessed the capacity for PUFA synthesis in males and females across the life course and specifically in immune cells.
Aim

The aim of this chapter is to determine whether the activity of the PUFA synthesis pathway, including the expression of the genes that operate in the pathway differs with age, gender and menopausal status in activated PBMCs.

Hypotheses

- The activity of the PUFA biosynthesis pathway in activated PBMCs is higher in females compared with males.
- The activity of the PUFA biosynthesis pathway in activated PBMCs is higher in pre- compared with post-menopausal females.
- The activity of the PUFA biosynthesis pathway in activated PBMCs decreases with increasing age.

To address these hypotheses the levels of individual n-3 PUFAs synthesised from [1-13C]18:3n-3 and the mRNA expression of FADS2, FADS1 and ELOVL5 were compared in Con A stimulated PBMCs from males and females of differing ages. Activation of PBMCs by Con A was confirmed by measuring the cell surface expression of the activation marker CD69. Analysis of ELOVL2 mRNA expression was not carried out as its expression was shown to be negligible to absent in activated PBMCs (Chapter 4).
7.2 Methods

7.2.1 PBMC isolation and measurement of cell specific surface markers

Volunteers were recruited as part of the EPUFA study as described in section 2.2. Recruitment aimed to ensure an even spread of ages for males and females across the life course. Anthropometric measurements were taken (section 2.2.5) and plasma TG, total cholesterol, HDL cholesterol and glucose concentration and blood total haemoglobin concentration were measured as detailed in section 2.3. PBMCs were isolated from lithium heparin blood (section 2.4) and the proportions of T-lymphocytes, B-lymphocytes and monocytes within the PBMC population were determined by measuring the expression of the cell surface markers CD3, CD19 and CD14, respectively, using flow cytometry (section 2.5). These markers were also analysed following cell culture.

7.2.2 PBMC culture and measurement of CD69 expression

Isolated PBMCs were cultured for 48 h in the presence of 5 µg/ml Con A and 20 µM [1-13C]18:3n-3 diluted 1/10 with unlabelled 18:3n-3 as described in section 2.6. PBMC activation was confirmed by measurement of CD69+ events by flow cytometry (section 2.7).

7.2.3 Measurement of n-3 PUFA synthesis

[13C]-label incorporation into specific n-3 PUFAs was measured by a combination of GC and GC-C-IRMS as detailed in section 2.12 with normalisation to total cell protein (section 2.13).

7.2.4 Measurement of mRNA expression

Total RNA was extracted from PBMC pellets as described in section 2.14.2. The mRNA expression levels of FADS2, FADS1 and ELOVL5 were measured by real-time RT-PCR as detailed in section 2.15.
7.3 Results

7.3.1 Study population characteristics

Table 7.1 shows the characteristics of the volunteer population (total EPUFA population) included in the experiments detailed in this Chapter. All participants were considered healthy based on these measurements and information given in screening questionnaires. Males and females did not significantly differ by age, BMI, plasma total cholesterol concentration or plasma glucose concentration. Females had significantly lower plasma TG and higher plasma HDL concentrations (both \( P < 0.0001 \)). As expected mean body weight and total haemoglobin were significantly lower in females (\( P < 0.0001 \)) and body fat percentage significantly higher than in men (\( P < 0.0001 \)). The proportions of T-lymphocytes (CD3\(^+\)), B-lymphocytes (CD19\(^+\)) and monocytes (CD14\(^+\)) within the PBMC population are shown for freshly isolated (pre-culture) and PBMCs cultured in the presence of Con A (post-culture + 5 \( \mu \)g/ml Con A), in Table 7.2. The proportion of T-lymphocytes was significantly higher in cultured PBMCs compared with freshly isolated cells in both males and females (\( P < 0.01 \)). The proportion of B-lymphocytes decreased following culture but only reached statistical significance in females (\( P < 0.01 \)) where monocytes were significantly lower in both males and females (\( P < 0.001 \)).

<table>
<thead>
<tr>
<th>Table 7.1. Characteristics of total EPUFA study population</th>
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<tbody>
<tr>
<td>Characteristic</td>
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<tr>
<td>-----------------------------------------------------------</td>
</tr>
<tr>
<td>Age (years)*</td>
</tr>
<tr>
<td>Body weight (kg)</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
</tr>
<tr>
<td>Body fat (%)</td>
</tr>
<tr>
<td>Plasma TG (mmol/l)</td>
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<tr>
<td>Plasma total cholesterol (mmol/l)</td>
</tr>
<tr>
<td>Plasma HDL cholesterol (mmol/l)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
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<tr>
<td>Total haemoglobin (g/l)</td>
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</table>

Data are mean ± SEM; *data are mean [range]; Male n = 31, Female n = 37. P values are for differences between females and males determined using an independent-samples t-test.
Table 7.2. PBMC phenotypes

<table>
<thead>
<tr>
<th>Cell phenotype</th>
<th>Pre-culture</th>
<th>Post-culture + 5 µg/ml Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>CD3+</td>
<td>59.6 ± 2.0</td>
<td>62.9 ± 1.4</td>
</tr>
<tr>
<td>CD19+</td>
<td>7.6 ± 0.5</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td>CD14+</td>
<td>12.7 ± 0.9</td>
<td>11.0 ± 0.7</td>
</tr>
</tbody>
</table>

Data are mean ± SEM; Male n = 31, Female n = 37. Results are for positive events within the PBMC gate, measured by flow cytometry. Statistical comparisons were by one-way ANOVA with Tukey’s post hoc test. There were no significant difference between males and females when measured pre- or post-culture. Blue and red asterisks indicate means that were significantly from pre-culture measurements in separate male and female groups, respectively, for each cell phenotype (P < 0.01).

7.3.2 The effect of PBMC activation on CD69 expression

Activation of cultured PBMCs by Con A was confirmed by measuring the expression of CD69 on the cell surface. The mean proportion of PBMCs expressing CD69 was significantly higher in male (49.83%) compared with female (41.28%) volunteers (Figure 7.1).

![Figure 7.1. The effect of PBMC activation on CD69 expression.](image)

Graph of % CD69+ events within a PBMC gate for males (n = 31) and females (n = 37) showing mean ± SEM. Statistical analysis was by independent-samples t-test.
7.3.3 The relationship between age and n-3 PUFA synthesis

Multiple linear regression analysis was conducted to estimate the association between age and the amounts of individual synthesised n-3 PUFAs and also the sum of these measured n-3 PUFAs in males and females (Table 7.3). PBMCs are a heterogeneous mix of blood cell populations and different profiles of distinct cell types may lead to false discoveries. Multiple linear regression analysis provides a way of accounting for such potentially confounding variables that have been included in the model. Model 1 held the proportions of CD3+, CD19+ and CD14+ constant where Model 2 also held the proportion of activated cells (CD69+) constant. No significant associations were found in males or females in either model except for 20:3n-3 where model 2 predicted that an increase in age of one year is associated with an increase in $[1^{13}C]20:3n-3$ by 0.012 pmol/µg protein with 95% confidence that this value is between 0.000152 and 0.023. This would represent a difference in the amount of $[1^{13}C]20:3n-3$ between the youngest (20 years) and oldest (70 years) female volunteers of 0.6 pmol/µg protein, with the range of plausible values being between 0.008 and 1.15 pmol/µg protein.

7.3.4 The relationship between FADS2, FADS1 and ELOVL5 mRNA expression and age

The same multiple linear regression models described in section 7.3.3 estimated the association between age and the mRNA expression levels of FADS2, FADS1 and ELOVL5 (Table 7.4). Model 2 predicted that an increase in age of one year is associated with an increase in relative FADS2 mRNA expression by 0.012 for males, with 95% confidence that this value is between 0.003 and 0.022. This would represent a difference between the youngest (18 years) and oldest (71 years) male volunteer of 0.636 with the range of plausible values being between 0.159 and 1.166. There were no significant associations between age and FADS1 or ELOVL5 mRNA expression levels in males or females.
### Table 7.3. Multiple regression analysis of the relationship between n-3 PUFA synthesis and age

<table>
<thead>
<tr>
<th>n-3 PUFA</th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β (95% CI)</td>
<td>P</td>
<td>β (95% CI)</td>
<td>P</td>
<td>β (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>-0.018 (-0.055, 0.019)</td>
<td>0.324</td>
<td>0.008 (-0.040, 0.055)</td>
<td>0.748</td>
<td>-0.007 (-0.046, 0.031)</td>
<td>0.703</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>-0.012 (-0.028, 0.003)</td>
<td>0.119</td>
<td>0.009 (-0.004, 0.022)</td>
<td>0.157</td>
<td>-0.008 (-0.024, 0.009)</td>
<td>0.344</td>
</tr>
<tr>
<td>20:4n-3</td>
<td>-0.006 (-0.018, 0.006)</td>
<td>0.343</td>
<td>0.008 (-0.005, 0.022)</td>
<td>0.225</td>
<td>-0.002 (-0.015, 0.010)</td>
<td>0.710</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>-0.002 (-0.007, 0.003)</td>
<td>0.534</td>
<td>-0.002 (-0.006, 0.002)</td>
<td>0.386</td>
<td>-0.000 (-0.005, 0.005)</td>
<td>0.126</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>-0.001 (-0.005, 0.003)</td>
<td>0.683</td>
<td>-0.001 (-0.003, 0.002)</td>
<td>0.520</td>
<td>0.001 (-0.003, 0.004)</td>
<td>0.718</td>
</tr>
<tr>
<td>Total</td>
<td>-0.039 (-0.097, 0.020)</td>
<td>0.184</td>
<td>0.022 (-0.041, 0.086)</td>
<td>0.479</td>
<td>-0.017 (-0.075, 0.042)</td>
<td>0.561</td>
</tr>
</tbody>
</table>

*β* denotes the parameter estimate (estimated change in [1-13C]n-3 PUFA amount (pmol/µg protein) per increase in age of one year). ’Total’ sum of all measured [1-13C]n-3 PUFAs. Model 1 was adjusted for the proportions of CD3+, CD19+ and CD14+ cells and Model 2 included CD69+ cells as an additional covariate. *P < 0.05.

### Table 7.4. Multiple regression analysis of the relationship between FADS2, FADS1 and ELOVL5 mRNA expression and age

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β (95% CI)</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADS2</td>
<td>0.009 (-0.001, 0.018)</td>
<td>0.073</td>
<td>-0.004 (-0.017, 0.009)</td>
<td>0.549</td>
<td>0.012 (0.003, 0.022)</td>
<td>0.013*</td>
</tr>
<tr>
<td>FADS1</td>
<td>0.001 (-0.001, 0.003)</td>
<td>0.357</td>
<td>-0.001 (-0.005, 0.002)</td>
<td>0.377</td>
<td>0.002 (0.001, 0.005)</td>
<td>0.039</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>-0.014 (-0.043, 0.014)</td>
<td>0.311</td>
<td>-0.005 (-0.037, 0.027)</td>
<td>0.781</td>
<td>-0.011 (-0.21, 0.043)</td>
<td>0.502</td>
</tr>
</tbody>
</table>

*β* denotes the parameter estimate (estimated change in relative mRNA expression level per increase in age of one year). Model 1 was adjusted for the proportions of CD3+, CD19+ and CD14+ cells and Model 2 included CD69+ cells as an additional covariate. *P < 0.05.

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The effect of gender on n-3 PUFA synthesis

The amounts of individual [1-13C] labelled n-3 PUFAs were compared in Con A treated PBMCs from male and female volunteers (Figure 7.2). Statistical analysis by one-way ANCOVA with the proportions of CD3+, CD19+ and CD14+ cells and age as covariates indicated there were no significant differences between males and females. Inclusion of the proportion of activated cells (CD69+) as an additional covariate also returned no significant results. Adjusted means are not shown as there were no significant differences. Furthermore, there were no significant differences between males and females when the sum of individual [1-13C]18:3n-3 conversion products were analysed by ANCOVA with the covariates described above (data not shown).

![Figure 7.2. Comparison of n-3 PUFA synthesis in males and females.](image)
The amounts of individual [1-13C] labelled n-3 PUFAs in male (n = 30) and female (n = 36) volunteers. Data were analysed by two different one-way ANCOVA models, one with the proportions of CD3+, CD19+ and CD14+ cells and age as covariates and another with CD69+ cells as an additional covariate. Graphs show mean ± SEM (unadjusted).
7.3.6 The effect of gender on \textit{FADS2, FADS1} and \textit{ELOVL5} mRNA expression

The mRNA expression levels of \textit{FADS2, FADS1} and \textit{ELOVL5} were compared in Con A treated PBMCs from male and female volunteers (Figure 7.3). Statistical analysis by one-way ANCOVA with the same covariates detailed in section 7.3.5 indicated there were no significant differences between males and females.

![Figure 7.3](image)

**Figure 7.3. Comparison of \textit{FADS2, FADS1, and ELOVL5} mRNA expression in males and females.** Relative mRNA expression normalised to \textit{EIF4A2, 18S, SDHA} and \textit{RPL13A} in male (n = 31) and female (n = 36 for \textit{FADS2} and 35 for \textit{FADS1} and \textit{ELOVL5}) volunteers. Data were analysed by two different one-way ANCOVA models, one with the proportions of CD3+, CD19+ and CD14+ cells and age as covariates and another with CD69+ cells as an additional covariate. Graphs show mean ± SEM (unadjusted).

7.3.7 The effect of menopausal status on n-3 PUFA synthesis

Since female hormones have been shown to upregulate n-3 PUFA synthesis, the amounts of individual [1-\textsuperscript{13}C]n-3 PUFAs were compared in Con A treated PBMCs from pre- and post-menopausal females (Figure 7.4). Mean differences assessed by one-way ANCOVA adjusted for the proportion of CD3+, CD19+ and CD14+ cells returned no significant differences. Inclusion of CD69+ cells as an additional covariate also returned no significant results. Furthermore, there were no significant differences between pre- and post-menopausal females when the sum of individual [1-\textsuperscript{13}C]18:3n-3 conversion products were analysed by ANCOVA with the covariates described above (data not shown).
Figure 7.4. Comparison of n-3 PUFA synthesis in pre- and post-menopausal females.
The amounts of individual [1-13C] labelled n-3 PUFAs in pre-menopausal (n = 25) and post-
menopausal (n = 9) volunteers are shown. Data were analysed by two different one-way ANCOVA
models, one with the proportions of CD3+, CD19+ and CD14+ cells as covariates and another with
CD69+ cells as an additional covariate. Graphs show mean ± SEM (unadjusted).

7.3.8 The effect of menopausal status on FADS2, FADS1 and ELOVL5 mRNA expression

Differences in the mRNA expression levels of FADS2, FADS1 and ELOVL5 were compared in Con A
treated PBMCs from pre- and post-menopausal female volunteers (Figure 7.5). Analysis by one-way
ANCOVA including the covariates described in section 7.3.7 returned no significant differences
between females that were pre- or post-menopause.
Figure 7.5. Comparison of \textit{FADS2, FADS1, ELOVL5} and \textit{ELOVL2} mRNA expression in pre- and post-menopausal females.

Relative mRNA expression normalised to \textit{EIF4A2, 18S, SDHA} and \textit{RPL13A} in pre-menopausal (n = 25 for \textit{FADS2} and 24 for \textit{FADS1} and \textit{ELOVL5}) and post-menopausal (n = 9) volunteers. Data were analysed by two different one-way ANCOVA models, one with the proportions of CD3+, CD19+ and CD14+ cells as covariates and another with CD69+ cells as an additional covariate. Graphs show mean ± SEM (unadjusted).
7.4 Discussion

The findings from this chapter suggest that age, gender and menopausal status do not affect the level of n-3 PUFA synthesis or the mRNA expression of three key enzymes that function in the PUFA biosynthesis pathway in activated PBMCs.

There were no significant differences in the proportions of CD3+, CD19+ and CD14+ between male and female volunteers. Activation of PBMC cultures was confirmed by measuring expression of the activation marker CD69. The proportion of cells expressing CD69 was significantly higher in males compared with females. Differences in immune functions between males and females have been observed previously. For example, both humoral and cell mediated immunity have been reported to be more active in females than in males (385, 386). Furthermore, in a previous study using PHA stimulated human cells, the percentage of CD69+ T lymphocytes was reported to be significantly higher in female samples compared with males (387). Possible reasons for the conflicting findings could include the use of a different T-lymphocyte mitogen, the younger age range of volunteers (18-38), or the smaller number of volunteers (6 males and females) compared to the present study. Leave out differences in immune functions.

There were no differences in the amounts of n-3 PUFAs synthesised from [1-13C]18:3n-3 or FADS2, FADS1 or ELOVL5 mRNA expression levels between males and females. Furthermore, these measures did not differ between pre- and post-menopausal women. These findings disagree with earlier reports of greater activity of the PUFA synthesis pathway in females compared to males (95, 96, 99, 100) and in response to female hormones (102-104). However, there are several differences between the present study and the earlier studies reporting gender differences in n-3 PUFA synthesis. The previous studies measured plasma concentrations of labelled n-3 PUFAs following ingestion of stable isotope labelled 18:3n-3, where the present study used a culture system, which may explain the inconsistent findings. Plasma concentrations represent an indirect measure of 18:3n-3 conversion and therefore have limited precision, as they do not account for those synthesised PUFAs not released into the bloodstream or taken up by target tissues. The artificial environment provided by the present culture method is also a limitation and labelled n-3 PUFAs that may have been released into the culture media were not examined. Furthermore, neither procedure accounted for further metabolism of synthesised n-3 PUFAs into lipid signalling molecules. The previous studies measured the levels of stable isotope labelled n-3 PUFAs at regular intervals and over longer time-periods than the present study. In the studies by Burdge and Wootton, analysis was based on area under the curve of the time course plot of the plasma [13C] labelled fatty acids over 21 days (99, 100). Pawlosky et al., used a physiological compartmental model to determine the rate constant coefficients for the conversion of individual fatty acids from
plasma concentration time curves of deuterated n-3 PUFAs over 168 h (95). Therefore, the single measurement of [13C] labelled n-3 PUFAs after 48 h performed in the present study may not have been sufficient to detect gender differences. However, in a previous study 48 h provided an adequate culture period for measuring the effects of progesterone treatment on n-3 PUFA synthesis in HepG2 cells and FADS2 expression in HepG2 cells and primary hepatocytes (104). In addition to different study designs and analysis procedures the previous studies had substantially fewer participants, six and five males and females, in the studies by Burdge and Wootton, 2002 (99, 100) and Pawlosky et al., 2003 (95, 96), respectively compared to 31 males and 37 females in this study.

The gender differences reported in previous studies were largely explained by synthesis of 22:6n-3. The studies in young men and women reported 200-fold greater synthesis of 22:6n-3 in women compared to men yet only 2.5 fold greater synthesis of 20:5n-3 and no differences in 22:5n-3 (99, 100). Pawlosky et al., 2003 only found a significant gender difference in synthesis of 22:6n-3 from 22:5n-3, where the rate constant coefficient was 4-fold greater in women compared with men (95, 96). Interestingly, activated PBMCs did not synthesise 22:6n-3, which may provide another explanation for the discrepancy in findings. Another explanation for the different findings could be that regulation of n-3 PUFA synthesis in activated PBMCs may differ to other tissues. Furthermore, activated PBMCs showed the highest level of conversion to 20:3n-3 after 48 h, a product of the delta-8 desaturation pathway, which was not measured in the earlier studies.

Some outlying data points were observed in the gene expression data in Figure 7.3. The gene expression data for FADS2 males contained an outlier that had a relative expression value of 1.87; the next data point below had a value of 1.18. For ELOVL5 females, the observed outlier had a value of 6.75 whereas the next data point below was 3.29. The elevated mRNA expression measured in these individuals did not correspond to higher amounts of stable isotope labelled n-3 PUFAs and therefore could not explain higher synthesis in these individuals. However, higher mRNA expression does not necessarily translate to higher levels of corresponding proteins, the latter of which could be measured using the Western blot technique. There is a chance that these outliers may have been a product of experimental error and the experiment would ultimately need repeating in order to verify that they are true results.

Previous reports on the effect of age on the activity of the PUFA synthesis pathway have been conflicting with some reporting decreases where others no change (97, 100, 117-119). In this study, there was no significant effect of age on the amounts of synthesised n-3 PUFA except for 20:3n-3, which was predicted to increase with increasing age in females when the proportion of activated cells was kept constant. However, the model only predicted and increase of 0.012 pmol/µg protein
per year, which represents a difference of 0.6 pmol/µg protein between the youngest and oldest female volunteer. Similarly, there were no significant relationships between mRNA expression levels and age except for FADS2, which was predicted to be 0.012 units higher with each year of age in males when adjusted for the proportion of activated cells. This would be mean a difference of 0.636 units between the youngest and oldest male volunteers. However, whether these values are biologically relevant is not known. As mentioned earlier there were no differences in the amounts of [1-¹³C] labelled conversion products or the mRNA expression levels of FADS2, FADS1 or ELOVL5 between pre- and post-menopausal women. This complements an earlier study that reported no differences in desaturase function between pre- and post-menopause groups measured indirectly using ratios of substrates and products (119).

In summary, no differences in the activity of the PUFA synthesis pathway were found between males and females in activated PBMCs. Furthermore, menopausal status did not affect PUFA synthesis activity. Together this data suggests that female hormones are not important for the regulation of n-3 PUFA synthesis in activated PBMCs, which is contradictory to some findings in other tissues. However, the difference in results may also be explained by variations in study design or the lack of 22:6n-3 synthesis in activated PBMCs. Finally, there was no clear effect of age on capacity for 18:3n-3 conversion.
Chapter 8: Final Discussion
8.1 Activation of PBMCs induces upregulation of the PUFA synthesis pathway

The findings from this study showed that n-3 PUFA synthesis is activated in PBMCs stimulated with the T-lymphocyte mitogen Con A. This finding is consistent with an early study that demonstrated activation of the pathway in PBMCs treated with a different T-lymphocyte mitogen, PHA (147). In the present study, activation of PBMCs by Con A also significantly increased the mRNA expression of three genes (FADS2, FADS1 and ELOVL5) encoding enzymes involved in conversion of 18:3n-3 to longer chain metabolites, compared with untreated PBMCs. This complements previous findings in other tissues where increased activity of the PUFA synthesis pathway was associated with increased expression of the genes encoding the pathway enzymes (105, 213). Taken together, these findings suggest that Con A mediated upregulation of n-3 PUFA synthesis is controlled at the transcriptional level.

The most highly synthesised n-3 PUFA after 48 h culture was 20:3n-3, a product in the alternative delta-8 desaturation pathway. This provides the first direct evidence for synthesis of 20:3n-3 in mitogen stimulated PBMCs. The inability to detect 18:4n-3, the delta-6 desaturase conversion product of 18:3n-3, suggested that activated PBMCs might be using the alternative pathway as the main route for longer chain n-3 PUFA synthesis. However, a recent study in a human MCF7 cell system stably transformed with FADS2, showed that increasing the availability of 18:3n-3 resulted in accumulation of 20:4n-3 in preference to 18:4n-3, indicating rapid or coupled elongation of 18:4n-3 (22). Therefore, in the present study 18:4n-3 may have been synthesised but did not accumulate to the level where it could be detected. A delta-8 desaturation step would be required to feed 20:3n-3 back into the main pathway; whether this step was occurring in PBMCs was not experimentally determined. There is no molecular evidence for a specific gene that encodes delta-8 desaturase activity, but the Fads2 gene product has been shown to be able to delta-8 desaturate 20:3n-3 to 20:4n-3 in S. cerevisiae transformed with mammalian Fads2 (19). However, there has been no investigation into the ability of delta-6 desaturase to catalyse delta-8 desaturation in mammalian cells. There is conflicting evidence over the functioning and importance of delta-8 desaturation in mammalian tissues (36, 38, 39, 41, 42). A study in mice showed that metabolism of 20:3n-3 by elongation and delta-5 desaturation to 20:4(5,11,14,17) was much greater than delta-8 desaturation (33). Delta-5 desaturation of 20:3n-3 but not delta-8 desaturation has also been reported as an alternative route for n-3 PUFA synthesis in the K562 leukemic cell line, which lacks delta-6 desaturase activity (44).

Findings from the present study suggested that ELOVL5 was more highly expressed than FADS2 in activated PBMCs. In a previous study, heterologous expression of human ELOVL5 in S. cerevisiae revealed that it is capable of elongating 18:3n-3 to 20:3n-3 (26). Therefore, since in the present
study both ELOVL5 expression and 20:3n-3 synthesis were elevated, it is speculated that elongation of 18:3n-3 to 20:3n-3 in PBMCs is catalysed by elongase-5. Furthermore, potential competition between elongase-5 and delta-6 desaturase for the substrate 18:3n-3 may explain the prevalence of 20:3n-3 in activated PBMCs. These results indicate that conversion to 20:3n-3 could be important as an alternative route for n-3 PUFAs in situations where delta-6 desaturase is limited. However, if delta-8 desaturation is catalysed by delta-6 desaturase, conversion of 20:3n-3 to 20:4n-3 and downstream n-3 PUFAs in the main pathway would also be restricted, as demonstrated in K562 leukaemic cells that lack delta-6 desaturase activity (44).

There was no evidence for synthesis of 22:6n-3 in resting or activated PBMCs. Real-time RT-PCR analysis revealed the expression of ELOVL2 to be absent to negligible in activated PBMCs. This is consistent with limited expression of ELOVL2 in most human tissues including an apparent lack of expression in human leukocytes (388). Elongase-2 has been shown to be involved in the conversion of 22:5n-3 to 22:6n-3 by catalysing the elongation of 22:5n-3 to the 24 carbon intermediate 24:5n-3 (27). Therefore, the questionable expression of ELOVL2 mRNA may explain the lack of 22:6n-3 synthesis observed in PBMCs. Both elongase-2 and elongase-5 are capable of catalysing the elongation of 20:5n-3 to 22:5n-3 (389). However, the results from this study indicate that elongase-5 (the product of the ELOVL5 gene) is the main if not sole elongase enzyme responsible for PUFA elongation in activated PBMCs. The findings from this study have provided important mechanistic insights into the activity of the PUFA synthesis pathway in activated PBMCs, which are summarised in Figure 8.1.
Figure 8.1. Schematic diagram showing activity of PUFA synthesis pathway measured in PBMCs. Green arrows indicate components that increased in Con A stimulated compared to unstimulated PBMCs. Components that were not detected (ND), present at low levels (low) or not synthesised (indicated by a cross) are shown in red. Genes speculated to function at the corresponding step are shown in blue with a question mark. The pathway intermediates 24:5n-3 and 24:6n-3 were not measured.

8.2 DNA methylation changes in activated PBMCs

The findings discussed so far suggest that upregulation of n-3 PUFA synthesis in activated PBMCs is controlled at the transcriptional level. DNA methylation changes are important in regulating the expression of specific cytokine genes involved in T-lymphocyte activation (297-301). In general, low methylation of promoter regions is associated with actively transcribed genes, whereas high methylation is associated with transcriptional silencing (292, 293). However, in the present study, analysis of DNA methylation in the FADS2 promoter, covering a region that previously displayed plasticity in PBMCs (261), revealed extensive increases in methylation in activated PBMCs. The DNA methylation status of one CpG dinucleotide in the FADS1 promoter region was also significantly increased. These findings are contradictory to the general view that DNA methylation is a repressive epigenetic mark (292, 293).

The relationship between DNA methylation and gene expression is complex and there are several hypotheses that might explain the increased levels of both measured in this study. The finding could be attributable to the heterogeneous cell population if the methylation change occurred in a different cell type or subset to the source of the gene expression change, or if changes to the
proportion of different cell types passively altered the methylation status. Another explanation could be that the DNA methylation prevents the binding of an inhibiting factor such as a transcriptional repressor. In silico analysis, using MatInspector software, predicted that a number of transcription factors, previously reported to be involved in lymphocyte function, might bind across the differentially methylated in FADS2. Two of these transcription factors, DEC2 and MNT, are known transcriptional repressors (310, 335), although DEC2 has also been shown to activate genes in T-lymphocytes (311). Alternatively, DNA methylation of CpG dinucleotides may act to enhance binding of transcription factors as shown for C/EBPα and SP1 (235, 237). Finally, the increased methylation might be due to the presence of 18:3n-3 in the culture medium. In rodents, 18:3n-3 supplementation has been shown to induce FADS2 promoter methylation (259). Therefore, one theory is that increased DNA methylation, induced by 18:3n-3, attenuated the increase in FADS2 gene expression brought about by upregulation of a FADS2 transcriptional activator in stimulated PBMCs. This theory assumes that active methylation was increased under stimulating conditions as 18:3n-3 was added to both unstimulated and stimulated cultures.

A considerable fraction of genes with methylated proximal promoters display elevated expression and in many such cases transcription is initiated at a distal CpG island that functions as an alternative promoter (339). Given the extent of increased FADS2 methylation in PBMCs following Con A treatment, the use of an alternative transcription initiation site for FADS2 transcription in activated PBMCs was hypothesised. This hypothesis was tested using 5’RACE, which enabled amplification and subsequent sequencing of the 5’ ends of FADS2 mRNAs. The results indicated that there was no distinct difference in the FADS2 TSS between unstimulated and Con A stimulated PBMCs.

In addition to their promoter regions, the methylation status of specific CpG dinucleotides located in the intergenic region between the FADS1 and FADS2 genes were measured. The region analysed has been previously reported to display the histone modification signature of a putative enhancer (273). Furthermore, in the same previous study, the methylation status of a specific CpG dinucleotide (cg27386326) located in this putative FADS enhancer region was shown to correlate negatively with delta-6 and delta-5 desaturase activities albeit being measured using the surrogate product to precursor ratio (273). In the present study, the methylation levels of two CpG dinucleotides including cg27386326 and an adjacent CpG dinucleotide (113 bp downstream) were significantly lower in Con A stimulated compared with unstimulated PBMCs but only by 3.5 and 2.1 percentage points, respectively. It is questionable whether a change this small could explain the magnitude of change in FADS1 and FADS2 gene expression. Nevertheless, predicted binding sites for transcription factors, including SREBP1, provided further support for this region as a possible enhancer, although actual binding would need proving experimentally.
In summary, activation of PBMCs by Con A induced DNA methylation changes in the FADS2 and FADS1 promoter regions and a putative enhancer region situated between these genes, but it is not clear how these changes relate to the transcriptional regulation of PUFA synthesis.

8.3 Discussion of possible functions of PUFA synthesis in activated PBMCs

The activation of PUFA synthesis in PBMCs following Con A stimulation raises the question of its biological function. Activation of lymphocytes induces proliferation, which is accompanied by both phospholipid synthesis and remodelling of existing phospholipids in order to support new membrane formation (137-139). A recent study in CD4+ T-lymphocytes demonstrated that both fatty acid uptake and de novo fatty acid synthesis from glucose are required for robust proliferation after antigenic stimulation (146). Considering this, it was postulated that PUFA synthesis might serve a role in supporting lymphocyte proliferation. The intracellular dye, CFSE was used to trace the proliferation of lymphocytes in PBMC cultures treated with or without the delta-6 desaturase inhibitor SC-26196. Results suggested that inhibition of the PUFA pathway causes a small decrease in proliferation. This reflects a previously proposed function of n-3 PUFA synthesis in supporting membrane synthesis through fine-tuning membrane fatty acid composition (147). However, a limitation of the present study was that PUFA synthesis was only reduced by the inhibitor, not completely restricted and therefore enough synthesis may have been maintained to support proliferation. Furthermore, it is possible that PUFA synthesis is of greater importance when exogenous long chain PUFAs are limited. Consequently, the importance of PUFA synthesis in supporting lymphocyte proliferation remains unclear.

The suggestion that PUFA synthesis is required for proliferation conflicts with the evidence that both n-6 and n-3 PUFA inhibit lymphocyte proliferation (163, 164, 390, 391). This might be explained by concentration and time factors where a finely controlled timing and level of synthesis may serve to aid proliferation. This suggestion is supported by a study that showed lower concentrations of the n-6 PUFA 18:2n-6 stimulated lymphocyte proliferation where higher concentrations inhibited it (167). An alternative theory is that PUFA synthesis might be involved in modulating lymphocyte responses and therefore serves an immuno-regulatory function. The possible immune-regulatory role aligns with the widely reported anti-inflammatory effects of n-3 PUFAs. However, since 18:2n-6 is much more prevalent in the diet, the metabolism of n-6 PUFA may be more relevant. Modulation of lymphocyte responses by newly synthesised PUFAs might be mediated via their conversion to lipid signalling molecules such as eicosanoids and, in the case of n-3 PUFAs, resolvins. Eicosanoids, such as prostaglandin E$_2$, are known to have anti-proliferative effects. However, evidence suggests that inhibition of lymphocyte proliferation by PUFAs is independent of eicosanoids (392). Furthermore, lymphocytes have been reported to be poor
producers of eicosanoids (168) although an earlier study demonstrated PUFA synthesised by lymphocytes can be utilised by monocytes for eicosanoid synthesis (286).

Another way newly synthesised PUFAs may act to modulate lymphocyte proliferation is through their effects on plasma membrane organisation and fluidity. An increase in membrane fluidity is a characteristic part of lymphocyte activation and is associated with a change in membrane phospholipid fatty acid composition. Incorporation of PUFA from the culture media in stimulated lymphocytes has been shown to further increase membrane fluidity (40, 49) and it has been proposed that that this change in fluidity might be the mechanism through which these fatty acids inhibit lymphocytes responses (40, 49). Enrichment of PUFA in the plasma membrane also alters the lateral organisation of membrane signalling assemblies called lipid rafts. Several important processes involve lipid rafts including T-lymphocyte activation and signal transduction (204). n-3 PUFAs have been reported to disrupt efficient functioning of lipid rafts in T-lymphocytes and in turn suppress their activation (207).

In this study, both the amounts of synthesised n-3 PUFAs and the mRNA expression of FADS2, FADS1 and ELOVL5 positively correlated with the proportion of PBMCs expressing the activation marker CD69. This further confirmed that the PUFA synthesis pathway is upregulated in activated cells. Interestingly, recent evidence suggests that CD69 could be involved in down regulating immune responses, which contradicts early in vitro data that suggested CD69 exerts a proinflammatory function (393, 394). For example, CD69 has been demonstrated to modulate immune allergic responses through negative regulation of allergen induced T-cell effector responses (395). The biological functions of both CD69 and PUFA are pleiotropic and complex but a possible link between CD69 expression and PUFA synthesis may be a potentially productive research area to explore.

Finally, 20:3n-3, a product in the alternative delta-8 desaturation pathway, was the main n-3 PUFA synthesised in activated PBMCs. It is not known whether the delta-8 desaturation pathway functions as an alternative route for longer chain n-3 PUFA synthesis or whether the products of this pathway have their own distinct biological activities. At present, there is no evidence that 20:3n-3 and 20:2n-6, or their delta-5 desaturation conversion products 20:4(5,11,14,17) and 20:3(5,11,14), respectively, are metabolised directly to lipid signalling molecules. However, 20:2n-6 has been shown to modulate the production of inflammatory mediators, including increasing prostaglandin E₂ (PGE₂) and tumour necrosis factor-α (TNF-α) production, in murine macrophages (287). Furthermore, in another study, 20:2n-6 was found to be a potent inhibitor of leukotriene B₄ (LTB₄) binding to pig neutrophil LTB₄ receptors (288). Considering this, PUFAs synthesised in the
alternative delta-8 desaturation pathway, previously thought to be dead end products, may in fact have important functions in immune cells.

To summarise, newly synthesised PUFAs may serve many functions in activated lymphocytes, including supporting synthesis of new cell membranes to support proliferation, modifying the production and activity of inflammatory mediators, influencing the function of membrane associated proteins and signaling pathways by effecting membrane architecture and fluidity and by modulating gene expression. Furthermore, these targets are interconnected and therefore influence the activity of one another. T-lymphocytes are crucial regulators of the immune response and greater understanding of the role of PUFA synthesis in activated T-lymphocytes could ultimately lead to the discovery of potential targets for treatment of immune-cell mediated disorders. Figure 8.2 attempts to give an overview of the regulation and possible functions of PUFA synthesis in activated PBMCs.

![Diagram](image)

**Figure 8.2. Schematic summary of the regulation and possible functions of PUFA synthesis in PBMCs.**

The top half of the diagram (lighter blue) summarises the findings from the analysis of PUFA synthesis in Con A stimulated relative to unstimulated PBMCs in this study. The bottom half of the diagram (darker blue) depicts the speculated functions of newly synthesised PUFAs.
8.4 PUFA synthesis is dysregulated in a spontaneously dividing T-lymphocyte leukaemic cell line

The findings from this study revealed that n-3 PUFA synthesis was elevated in the spontaneously dividing Jurkat T-lymphocyte leukaemic cell line. Conversion of 18:3n-3 to 20:4n-3, 20:5n-3 and 22:5n-3 was substantially higher in Jurkat cells relative to stimulated PBMCs and the HepG2 liver carcinoma cell line, corroborating previous reports of elevated PUFA synthesis in Jurkat cells compared with other cell types (148). However, the present study showed for the first time that FADS2, FADS1 and ELOVL5 mRNA expression levels are also elevated in Jurkat cells compared with activated PBMCs and HepG2 cells. Together these findings suggest transcriptional dysregulation of PUFA synthesis in Jurkat cells. Furthermore, analysis of DNA methylation in the FADS2 promoter region revealed substantially lower methylation over a large number of CpG dinucleotides, between 667 bp and 1655 bp upstream of the reported FADS2 TSS, in Jurkat cells compared with activated PBMCs. Lower methylation was also measured for two CpGs dinucleotides in the FADS1 promoter region. Since high levels of gene expression are generally associated with low levels of promoter methylation, the present findings imply that transcriptional dysregulation of PUFA synthesis in Jurkat cells involves altered DNA methylation. Aberrant DNA methylation is a common feature of cancerous cells (374). Promoter hypomethylation and increased gene expression have been reported for specific genes in various cancers including the BCL2 and HOXII genes in leukaemia (375, 376). In the present study, lower DNA methylation was also measured for a CpG dinucleotide located in the putative FADS enhancer region (273) in Jurkat cells compared to stimulated PBMCs, further supporting a possible role of this region as an enhancer of FADS transcription.

Detection of enriched peaks corresponding to 18:4n-3 and 18:2n-6 in GC-C-IRMS analysis confirmed the classical pathway was functioning in Jurkat cells. Enrichment in 20:3n-3 and 20:2n-6 was also detected in Jurkat cells, indicating that as in stimulated PBMCs, at least part of the delta-8 desaturation pathway was also operating. Inhibition of the delta-6 desaturase enzyme increased the synthesis of these PUFAs. This may be due to there being more 13:3n-3 and 18:2n-6 available for conversion, supporting the hypothesis that the alternative delta-8 desaturation and classical pathways are in competition. Alternatively, as demonstrated in yeast transformed with mammalian Fads2, delta-8 desaturation might be catalysed by the delta-6 desaturase enzyme. Therefore, if this same activity were present in Jurkat cells, inhibition of the delta-6 desaturase enzyme would explain the accumulation of 20:3n-3 and 20:2n-6 as a result of decreased conversion to 20:3n-6 and 20:4n-3, respectively. There was a low level of 22:6n-3 synthesis in Jurkat cells. However, the occurrence of this became questionable when an experiment showed that increasing the concentration of 18:3n-3 in the culture medium increased the levels of n-3 PUFAs upstream of 22:6n-3 but not 22:6n-3 itself. The enrichment measured in 22:6n-3 might be explained by carryover from the large
enriched 22:5n-3 peak beside the 22:6n-3 peak in GC-C-IRMS analysis. Questionable 22:6n-3 synthesis in Jurkat cells and also minimal ELOVL2 mRNA expression aligns with data from PBMCs and suggests possible truncation of the pathway at 22:5n-3 in T-lymphocytes.

Since Jurkat cells are a highly proliferating cell population, it was hypothesised that PUFA synthesis might be important for supporting the formation of new membranes in dividing cells. However, inhibition of the pathway using the delta-6 desaturase inhibitor SC-26196 had a minimal effect, decreasing cell number by 6%. This may have been because the concentration of inhibitor did not completely inhibit PUFA synthesis and sufficient levels were maintained to support growth. Alternatively, as discussed for PBMCs, the pathway may be serving additional functions to merely supporting cell growth. However, given the magnitude of PUFA synthesis in Jurkat cells and the high level of proliferation, the theory that PUFA synthesis might be involved in modulating the proliferative response becomes doubtful. However, the actions of newly synthesised PUFA in cancerous cells may not be comparable to normal cells. The high activity of the PUFA synthesis pathway in Jurkat cells reflects the dysregulated de novo fatty acid biogenesis from two carbon precursors in cancer cells in general (352). Elucidation of the functional significance of PUFA synthesis in Jurkat could be important for understanding how the activity of this pathway relates to the perpetuation of cancer cells.

8.5 The effect of age and gender on PUFA synthesis in activated PBMCs

In this study, the activity of the PUFA synthesis pathway in Con A stimulated PBMCs was compared between males and females across the life course. The activation of the pathway following stimulation clearly indicates its importance in the immune response but no previous studies have looked at the effect of age and gender on PUFA synthesis capacity in activated PBMCs. The amounts of n-3 PUFA synthesised from [1-13C]18:3n-3 were not found to differ between males and females. There were also no gender differences in the mRNA expression levels of FADS2, FADS1 and ELOVL5. Furthermore, neither of these measures differed between pre- and post-menopausal women. These findings disagree with earlier reports of greater activity of the PUFA synthesis pathway in females compared to males (95, 96, 99, 100) and in response to female hormones (102-104). Several factors may explain the difference in findings between the present and earlier studies. A major difference is represented by the study design; earlier studies measured concentrations of labelled n-3 PUFA in plasma following ingestion of labelled 18:3n-3 whereas the present study used a closed culture system. The former does not account for those synthesised PUFAs not released into the bloodstream or taken up by target tissues and the latter is an artificial environment, which may have had confounding effects. Secondly, the previous studies measured the levels of stable isotope labelled n-3 PUFAs at regular intervals over time-periods of 7 days (95)
and 21 days (99, 100) compared to a single measurement after 48 h in the present study. Finally, activated PBMCs were not found to synthesise 22:6n-3 yet gender differences in n-3 PUFA synthesis reported in previous studies were largely attributable to the synthesis of this fatty acid (95, 96, 99, 100) offering a further explanation for the discrepancy in findings. Despite these explanations, the results from this study may indicate that the mechanisms controlling n-3 PUFA synthesis are different in activated PBMCs compared with other tissues. Furthermore, it is not known whether the activity of alternative delta-8 desaturation pathway is influenced by hormonal factors.

The effect of age on n-3 PUFA synthesis is less clear with different studies yielding conflicting results (97, 100, 117-119). This study showed no clear effects of age on n-3 PUFA synthesis or FADS2, FADS1 or ELOVL5 mRNA expression. However, when the proportion of activated cells were held constant, multiple linear regression analysis predicted a small increase in 20:3n-3 synthesis (0.012 pmol/µg protein per one year increase in age) in females and FADS2 mRNA expression (0.012 units per one year increase in age) in males. Whether these values are biologically relevant is not known and overall neither gender nor age appeared to influence capacity of n-3 PUFA synthesis in activated PBMCs.

8.6 Thesis limitations

A limitation of this study that was relevant to all downstream analyses was the use of a heterogeneous cell population (PBMCs) as it was unclear which cell types or sub-types were the source of detected differences. The use of purified populations of cells would have enabled resolution of the cell types responsible for measured changes. However, the large volume of blood and time required for purification of single cell populations were not viable in this study. Furthermore, a vast number of publications have reported findings from mitogen treated PBMC populations as a model for lymphocyte activation.

The culture period of 48 h may have been another limitation in the study design. A 48 h time period was chosen because of previous work in our laboratory (63) and was sufficient to measure upregulation of PUFA synthesis in stimulated compared to unstimulated cells. However, a 48 h culture period only provided a snapshot of PUFA synthesis capacity and might not have been the optimal timeframe for analysis of gender and age differences. Nonetheless, in our previous study, a 48 h culture period was sufficient to measure the effect of progesterone treatment on the activity of the PUFA synthesis pathway in HepG2 cells and FADS2 mRNA expression in primary hepatocytes (104). Measurements at regular intervals over a longer time-period would provide a more complete assessment of the activity of the PUFA synthesis pathway. However, as before, the volume of blood required for this level of analysis was not feasible in this study.
A limitation relating to the analysis PUFA synthesis in pre- and post-menopausal females was the number of post-menopausal females recruited (nine) was less than half the number of pre-menopausal females (twenty-five). This represents a general limitation with recruiting enough volunteers within the timeframe of the study and older individuals proved more difficult to recruit. Despite this, a major advantage of the present study as a whole was the much higher number of participants for comparisons of n-3 PUFA synthesis in unstimulated and stimulated PBMCs and in males and females compared with earlier studies (95, 96, 99, 100, 396). Participants in the study were asked to limit consumption of oily fish and to cease any consumption of fish-oil supplements prior to the study. Compliance has not been verified but this could be done by analysing red blood cell fatty n-3 PUFA content as a biomarker of intake (76).

A major limitation when measuring the effect of inhibiting delta-6 desaturase on lymphocyte proliferation was the maximum concentration (200 nM) of the inhibitor (SC-26196) used in the experiments did not completely restrict PUFA synthesis, only reduced it. Higher concentrations of SC-26196 were avoided to prevent non-specific inhibition of desaturase activities. An alternative mechanism to inhibit the pathway using siRNA to knockdown FADS2 expression could be explored in future work.

GC-C-IRMS offers a highly sensitive method for directly measuring the synthesis of labelled n-3 PUFAs. However, analysis of 22:6n-3 synthesis in Jurkat cells revealed that the detected stable isotope enrichment in 22:6n-3 may have been a product of carryover from the large neighbouring 22:5n-3 peak. This has been previously reported as a limitation of GC-C-IRMS, where isotope measurements for compounds eluting immediately after enriched compounds were significantly affected due to carryover effects (371). This issue could be resolved by running the samples on a low polarity phase, 5% diphenyl/95% dimethyl polysiloxane capillary column (Rtx®-5, Thames Restek) that reverses the order in which the fatty acids are eluted.

S’RACE analysis was conducted to characterise the FADS2 TSS. Initially this was carried out using the S’RLM RACE kit which has the advantage of being specific for mature capped mRNAs. Unfortunately, this kit resulted in formation of an artefact, which appeared consistent since the same artefact was observed by another researcher. Therefore, S’RACE was carried out using a different kit (SMARTer™ RACE). Successful RACE products were obtained but the SMARTer™ RACE kit does not guarantee specificity for full length cDNAs. Eleven independent clones containing the gene specific inserts were sequenced (the kit recommends picking at least 8-10 different clones) in order to obtain the maximum amount of sequence at the 5’end.
8.7 Future work

The research presented in this thesis has identified a number of novel findings in relation to the characterisation and regulation of PUFA synthesis in activated PBMCs. Given more time, future experiments could build on the present findings to gain further understanding of the mechanistic and functional aspects of this pathway in PBMCs.

A number of experiments could help answer the unresolved mechanistic questions arising from this thesis. Firstly, the identity of the enzyme responsible for catalysing the elongation of 18:3n-3 to 20:3n-3 in activated PBMCs is not known but was speculated to be elongase-5. To verify this, siRNA could be introduced into activated PBMCs to silence ELOVL5 gene expression and synthesis of 20:3n-3 from 18:3n-3 could then be compared with activated PBMCs in which the ELOVL5 gene is functional. Secondly, whether 20:3n-3 was converted to 20:4n-3 and therefore fed back into the classical pathway remains unknown. This could be investigated by incubating cells with stable isotope labelled 20:3n-3 and measuring enrichment in 20:4n-3 and downstream conversion products. Although retro-conversion of 20:3n-3 to 18:3n-3 would have to be accounted for. Conversion of 20:3n-3 by delta-5 desaturation to the unusual product 20:4(5,11,14,17) could also be investigated using stable isotope labelled 20:3n-3. Lastly, to establish whether limited ELOVL2 expression underlies the lack of 22:6n-3 synthesis observed in PBMCs, cells could be incubated with downstream pathway intermediates (24:5n-3 and 24:6n-3) to determine whether 22:6n-3 synthesis is restored.

Higher n-3 PUFA synthesis and FADS2, FADS1 and ELOVL5 mRNA expression in stimulated compared with unstimulated PBMCs strongly indicates that upregulation of the pathway involves increased levels of the delta-6 and delta-5 desaturase and elongase-5 enzymes. However, mRNA expression levels do not always correlate with the expression of the correspondent protein. The Western blot technique would enable confirmation of increased protein levels. This technique could also be used to verify whether ELOVL5 was the most highly expressed pathway enzyme in activated PBMCs.

Functional characterisation of the putative FADS enhancer region represents an interesting area for future work. One approach would be to clone the enhancer region into a reporter plasmid construct containing a minimal promoter and then introduce the construct into cells for functional analysis. The effect of the putative enhancer on the activity of the minimal promoter could be tested by comparing the enzymatic activity of the reporter gene in cells transfected with the reporter construct either with or without the putative enhancer element. However, a major limitation of this technique is there is always the potential that the reporter construct will not completely recapitulate the normal functioning of a given promoter or enhancer.
Reporter gene assays can also be utilised to investigate the effect of methylation in promoter and/or enhancer regions on gene expression. A common method is to add the promoter or enhancer elements to a CpG dinucleotide free reporter vector such as PCpGL and methylate the whole construct. The absence of CpG dinucleotides in the plasmid backbone limits in vitro CpG methylation to the inserted CpG containing elements. Methylated or unmethylated constructs can then be transfected into cells and the expression of the reporter gene compared.

The putative FADS enhancer region was previously reported to have the histone modification signature of an enhancer (273). However, this analysis was carried out using ENCODE regulatory tracks and such predictive methods do not always translate universally across cell types. Therefore, in future experiments histone modifications in the putative FADS enhancer could be profiled experimentally, for example in unstimulated and stimulated PBMCs, using Chromatin Immunoprecipitation (ChIP). ChIP is a procedure used to examine protein interactions with DNA. When examining histone modifications, antibodies against specific histone marks are used to isolate the protein-DNA complex of interest and after removal of crosslinks the precipitated DNA can be quantified by real-time RT-PCR. Histone modifications associated with active transcription include histone H3 acetylation and di- or tri-methylation of histone H3 at lysine 4 (H3K4) whereas H3K9 methylation is associated with silent genes. Specifically, active enhancers are marked by monomethylation of H3K4 and H3K27 acetylation (397). In addition to profiling histone modifications, computational predictions of transcription factor binding sites can be experimentally tested using ChIP. Furthermore, binding of transcription factors to, for example, the FADS2 promoter could be measured under different experimental conditions such as in unstimulated and stimulated PBMCs.

Another technique, named the electrophoretic mobility shift assay (EMSA), has been widely used to study DNA-protein interactions. EMSA could be used to investigate the binding of transcription factors to the differentially methylated region in the FADS2 and FADS1 promoters and the putative FADS enhancer region. This technique is based on the principle that complexes of protein and DNA migrate more slowly than free linear DNA fragments through a non-denaturing polyacrylamide gel. The assay is performed by incubating a purified or complex mixture of proteins, such as a nuclear extract, with a labelled DNA fragment (probe) containing the putative protein binding site. Two main approaches are used to determine the specificity of binding and the identity of the protein involved: competition with unlabelled competitor DNA and incubation with antibodies specific to the putative DNA binding protein (antibody supershifts). Furthermore, methylated and unmethylated probes can be employed to determine the effect of methylation on transcription factor binding.
An important question that remains unanswered is the biological function of activated PUFA synthesis in stimulated PBMCs. One hypothesis is that newly synthesised PUFAs are channelled into production of lipid signalling molecules such as eicosanoids and resolvins. To test this hypothesis, stimulated PBMC cultures could be treated with stable-isotope labelled 18:2n-6 and 18:3n-3 and potential enrichment in eicosanoids or resolvins measured using mass spectrometry. This hypothesis could be further explored by comparing levels of eicosanoids and resolvins in activated PBMCs where the PUFA synthesis pathway is functional, with activated PBMCs where the pathway has been inhibited. Finally, as mentioned earlier, development of siRNA techniques might permit greater inhibition of the PUFA pathway, which would enable further characterisation of the importance of the pathway in supporting lymphocyte proliferation.
References


Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta. 2011;1812(8):1007-22.


Appendix A
### A.1 Table of reagents and products

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<td>Thermo Fisher Scientific, UK</td>
</tr>
<tr>
<td>Zymo Research EZ-96 DNA methylation-Gold™ Kit</td>
<td>Cambridge Bioscience, UK</td>
</tr>
<tr>
<td>Zymoclean™ Gel DNA Recovery Kit</td>
<td>Cambridge Bioscience, UK</td>
</tr>
</tbody>
</table>

A.2  Cell culture

A.2.1  Maintenance media

**PBMC medium:**

RPMI-1640 supplemented with 2 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. This media was supplemented further with 5% (v/v) autologous plasma or 10% (v/v) fetal bovine serum (FBS) for PBMCs isolated from volunteers’ lithium heparin treated blood and purchased cryopreserved PBMCs, respectively.

**Jurkat cell medium:**

RPMI-1640 medium supplemented with 2 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin and 10% (v/v) fetal bovine serum (FBS).
**HepG2 medium:**

Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FBS.

**A.2.2 Cell culture treatment compounds**

**Stable isotope labelled linoleic acid ([U-13C]18:2n-6):**

10 mM stock solution was made for both [U-13C]18:2n-6 (Sigma-Aldrich) and unlabelled 18:2n-6 (Sigma-Aldrich) in ethanol. These were then diluted to 1 mM in the appropriate maintenance medium. Labelled 18:2n-6 was then diluted 60-fold with unlabelled 18:2n-6 and added to the cell culture media at the appropriate concentration always ensuring that the percentage of ethanol in the final cell culture medium did not exceed 0.1%. The 60-fold dilution factor was determined experimentally (see section 2.12.5.3).

**Stable isotope labelled alpha-linolenic acid ([1-13C]18:3n-3):**

1 ml of ethanol was added to a vial containing 10 mg [1-13C]18:3n-3 (Campro Scientific) and vortexed. From this a 1 mM stock was then made in the appropriate growth medium for the cells being treated. A 1 mM stock of unlabelled 18:3n-3 (Sigma-Aldrich) was prepared in the same way. The 1 mM [1-13C]18:3n-3 was then diluted 10-fold with 1 mM unlabelled 18:3n-3 and added to the cell culture medium at the appropriate concentration always ensuring that the percentage of ethanol in the final cell culture medium did not exceed 0.1%. The 10-fold dilution factor was calculated from the 60-fold required for [U-13C]18:2n-6.

**Concanavalin A**

The T cell mitogen concanavalin A (ConA) was dissolved in PBMC medium to make a 0.5 mg/ml stock and added to cells at a final concentration of 5 µg/ml.

**SC-26196**

A 10 mM stock of the delta-6 desaturase inhibitor was prepared in DMSO. A series of 10-fold dilutions, the first in DMSO and the second and third in the appropriate maintenance media, were made to achieve a 10 µM stock solution. This was then added directly to the cell culture medium at the following concentrations: 50 nM, 100 nM, 150 nM and 200 nM.
A.3 Instrument settings for flow cytometry analyses

<table>
<thead>
<tr>
<th>Detector</th>
<th>Voltage</th>
<th>AmpGain</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application: PBMC Phenotyping and CD69 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSC</td>
<td>E00</td>
<td>2.55</td>
<td>Linear</td>
</tr>
<tr>
<td>SSC</td>
<td>407</td>
<td>3.0</td>
<td>Linear</td>
</tr>
<tr>
<td>FL-1</td>
<td>492</td>
<td>1.0</td>
<td>Log</td>
</tr>
<tr>
<td>FL-2</td>
<td>628</td>
<td>1.0</td>
<td>Log</td>
</tr>
<tr>
<td>FL-3</td>
<td>150</td>
<td>1.0</td>
<td>Log</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compensation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL-1 –</td>
</tr>
<tr>
<td>FL-2 –</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Application: CFSE proliferation and LIVE/DEAD red viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSC</td>
</tr>
<tr>
<td>SSC</td>
</tr>
<tr>
<td>FL-1</td>
</tr>
<tr>
<td>FL-2</td>
</tr>
<tr>
<td>FL-3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compensation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL-1 –</td>
</tr>
<tr>
<td>FL-2 –</td>
</tr>
</tbody>
</table>

A.4 Coefficients of variation

A.4.1 COBAS MIRA auto-analyser

<table>
<thead>
<tr>
<th></th>
<th>TG (mmol/l)</th>
<th>Total Cholesterol (mmol/l)</th>
<th>HDL Cholesterol (mmol/l)</th>
<th>Glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low QC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>40</td>
<td>30</td>
<td>11</td>
<td>70</td>
</tr>
<tr>
<td>Mean</td>
<td>1.28</td>
<td>2.71</td>
<td>0.89</td>
<td>4.97</td>
</tr>
<tr>
<td>SD</td>
<td>0.06</td>
<td>0.13</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.90</td>
<td>4.87</td>
<td>3.71</td>
<td>1.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>High QC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>SD</td>
</tr>
<tr>
<td>CV (%)</td>
</tr>
</tbody>
</table>
A.4.2 GC-C-IRMS δ-value

The δ-value (raw data) was monitored for unenriched and [^{13}C] enriched 18:3n-3 for all GC-C-IRMS runs to calculate an inter-assay CV. In a separate experiment, the δ-value intra-assay CV was measured for a [^{13}C] enriched 18:2n-6 sample diluted to achieve different peak areas.

<table>
<thead>
<tr>
<th></th>
<th>Unenriched 18:3n-3</th>
<th>Enriched 18:3n-3</th>
<th>Enriched 18:2n-6 (peak area of 1 to 2)</th>
<th>Enriched 18:2n-6 (peak area &lt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>14</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mean (δ-value)</td>
<td>-32.43</td>
<td>131.53</td>
<td>276.93</td>
<td>245.83</td>
</tr>
<tr>
<td>SD</td>
<td>1.56</td>
<td>7.86</td>
<td>13.08</td>
<td>23.35</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.80</td>
<td>5.98</td>
<td>4.72</td>
<td>9.50</td>
</tr>
</tbody>
</table>

A.5 Agarose gel electrophoresis DNA ladders

Thermo Scientific GeneRuler 1 kb Plus (left) and 100 bp Plus (right) DNA ladders. Images are taken from the product manual and show the size of the DNA reference bands.
Appendix B
Title of study: Epigenetic regulation of omega-3 polyunsaturated fatty acid synthesis (The ‘E-PUFA’ study)

We would like to invite you to take part in our research study. Before you decide whether or not you wish to take part it is important that you understand why the research is being done and what the research will involve. You are free to decide whether or not to take part in this study. Please read the information carefully and discuss it with others if you wish and ask us if there is anything that is not clear, or if you would like more information.

What is the purpose of this study?

Long chain omega-3 fatty acids are believed to be beneficial to human health. They can be obtained by eating oily fish. Humans can also make their own long chain omega-3 fatty acids in the body from alpha-linolenic acid (ALA), a fatty acid that can only be obtained from the diet, for example by eating vegetable oils. Since intake of oily fish is relatively low in the UK, this production of long chain omega-3 fatty acids from ALA could be important for meeting nutritional requirements. It has been found that increasing levels of long chain omega-3 fatty acids can help promote healthy ageing. However, there is limited information about our capacity to produce these fatty acids throughout our lives. It has been found that women have a greater ability to make long chain omega-3 fatty acids than men but we do not fully understand the biological mechanisms behind this.

We want to find out if the capacity of men and women to make long chain omega-3 fatty acids changes with age. If it does then we want to find out how this happens. Specific genes can make proteins called enzymes that are responsible for making long chain omega-3 fatty acids. In order to do this these genes get copied to a molecule called RNA which is then used by cells to build the enzymes. We will be measuring the amount of RNA produced by your cells to see if this relates to any changes in the synthesis of omega-3 fatty acids. We will also extract DNA from your cells in order to look at a mechanism called epigenetic regulation, which is a process that can finely tune the amount of protein that is made by our genes.
This work is important as it could help us to understand how nutritional requirements for long chain omega-3 fatty acids may change for men and women throughout their lives. This work could also help us understand how the production of these important fatty acids in the body is regulated. In this study we will be measuring long chain omega-3 fatty acids in blood and in cheek (buccal) cells.

**Can I take part?**

To take part in this study you are required to be a White, Caucasian, male or female, aged between 18 and 71 years, healthy and have a BMI between 18.5 and 30. You will not be able to take part if any of the following apply to you:

- You are pregnant
- You are currently taking the oral contraceptive pill
- You are receiving hormone replacement therapy
- You have type 1 or type 2 diabetes
- You are a smoker
- You are male and consume more than 28 units of alcohol per week or female and consume more than 21 units of alcohol per week
- You consume more than one portion of oily fish per week
- You consume fish oil capsules (consumption would have to be terminated 3 months prior to the start of the study if you wish to take part)

**Do I have to take part?**

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. However, you will still be free to withdraw at any time, and without giving a reason. If you do withdraw, you will be asked whether you want us to destroy any samples you have given as part of the study.

**What would taking part involve?**

Screening and study appointment:

If you decide that you would like to participate in this study you will have to attend a screening appointment before commencing the study. Prior to the screening appointment we will send you a basic health and diet questionnaire and you will need to complete and return part A of the questionnaire before coming for your appointment. You will need to arrive at your appointment fasting (nothing to eat since 10pm the night before, and only water to drink). You will be introduced to the study team and we will discuss the project with
you. You will be given the opportunity to ask any questions arising from the discussion or this information sheet. At this point, if you are happy with all aspects of the study and still wish to take part, we will ask you to sign a consent form.

If your consent is given, your study appointment will follow immediately after your screening appointment. We will measure your height, weight, blood pressure and your percentage body fat. You will be introduced to a research nurse or doctor who will take a 40-50 ml blood sample. You will also be asked to provide a cheek cell sample, which involves gently brushing the inside of your cheek with a brush similar to a toothbrush. The samples you give will be used to measure omega-3 fatty acids levels in your cells and the ability of your cells to synthesise these fatty acids. We will also measure blood glucose, triglyceride and cholesterol and haemoglobin using the blood sample you have given. A copy of the results will be sent to you and your GP. The study appointment will take place at the Cedar Centre, Royal Surrey County Hospital. The length of the visit should be approximately 1 hour. We will give you a drink and a snack to eat before you leave.

**How will the data collected be stored?**

All experimental data will be stored securely according to the data protection act 1998 on the password protected university servers (for a period of 10 years) and any information you give will be stored separately via a fully anonymous link. You will be given a trial number following recruitment. Sensitive information will not be stored on either personal home computers or disks. Electronic data sets will continue to be stored and accessed following completion of the study, however, your paper records and sensitive information which could identify you will be destroyed on completion of the study.

**What will happen to the samples I give?**

If you consent, we would like to keep any unused blood samples for further research related to the project. This could be another student project, for example. The samples will be used in a completely anonymised way and any use will have an appropriate ethical review. DNA samples will not be used for further research projects.
What are the possible benefits of taking part?

You will not receive any direct benefit from taking part in the study. However, you will receive a health check. Knowledge gained from this study will help our research and may ultimately be of use to other researchers and to consumers.

What are the possible disadvantages and risks of taking part?

There is a very small chance of bruising at the site of insertion of the needle for collection of blood samples. This risk will be minimised by using fully trained members of staff. You may feel a little faint when having your blood sample taken. If this has happened to you before then please inform us.

What will happen if anything goes wrong?

Any complaint or concern about any aspects of the way you have been dealt with during the course of the study will be addressed. If you have any concerns about any aspect of the study please contact:

Charlene Sibbons
Tel: 01483 688642
Email: c.sibbons@surrey.ac.uk

Alternatively, you can contact the Patient Advice and Liaison Service (PALS):

PALS at Royal Surrey County Hospital Egerton Road, Guildford GU2 7XX
Tel: 01483 402757
Email: rsc-tr.PALS@nhs.net

Will my taking part in this study remain confidential?

Your GP will be notified that you are participating in this study, but any information that is collected about you during the course of the research will be coded with a study number and will be kept strictly confidential.
What will happen to the study results?

We will inform you of the overall results from the study. The results of this research study may be presented at scientific meetings or published in a scientific journal. This may not happen until 1 or 2 years after the study is completed and you will not be identified in any of these presentations or publications. If you contact the researchers in the future we will let you know where you can obtain a copy of the published results.

Will I be reimbursed for my time?

In recognition of your time and commitment you will be paid an honorarium of £25.

Who is organising and funding the study?

This study has been organised by the University of Surrey and is funded by the Biotechnology and Biological Sciences Research Council Doctoral Training Partnership (BBSRC DTP) and the Rank Prize Fund.

Who has reviewed the study?

This study has been reviewed by the University of Surrey ethics committee and the NHS ethics review process.

Contact for further information

Charlene Sibbons
Tel: 01483 688642
Email: c.sibbons@surrey.ac.uk

Thank you for reading this information sheet. If you are interested in being involved in the study please contact the study team to arrange a screening appointment.
Screening Questionnaire

Title of study: Epigenetic regulation of omega-3 polyunsaturated fatty acid synthesis
(The ‘E-PUFA’ study)

Section A: To be completed by the participant

1. Participant Details

Name: 
Address: 

Contact telephone numbers: Day ........................................ Evening .................................
Best time to phone/on which number? 

Email address: 
Next of kin contact name: 
Next of kin telephone number: 
GP Name: 
GP Address: 

Date of birth: 
Age: 
Ethnicity: 
Occupation: 

2. Health

What is your approximate weight? 

Has your weight varied by more than half a stone (3kg) in the past 3 months? 

Yes ☐ No ☐
Do you have any of the following conditions?  

I. Diabetes  
   [ ] Yes  [ ] No

II. Liver disease  
   [ ] Yes  [ ] No

III. Renal disease  
   [ ] Yes  [ ] No

IV. Endocrine disorders  
   [ ] Yes  [ ] No

Are you currently taking any prescribed medication?  
   [ ] Yes  [ ] No

If YES, please give details ........................................................................................................................................

........................................................................................................................................................................

........................................................................................................................................................................

Do you smoke?  
   [ ] Yes  [ ] No

How many alcoholic drinks do you have on average per week? .................................................................

3. Diet  

Do you eat fish?  
   [ ] Yes  [ ] No

If yes, which types of fish do you eat (i.e. oily - salmon, mackerel, tuna, kipper or white - cod, bass, halibut, bream, sole)?  

........................................................................................................................................................................

........................................................................................................................................................................

How often do you eat each of these (i.e. number of times per week, month or year)?  

........................................................................................................................................................................

........................................................................................................................................................................
Are you currently consuming any fish oil, cod liver oil or omega-3 supplements?  

Yes  No  

4. If female  

Are you pregnant?  

Yes  No  

Are you taking the contraceptive pill?  

Yes  No  

Are you receiving hormone replacement therapy?  

Yes  No  

What was the approximate date of your last menstrual cycle?  

5. Other research studies  

Are you taking part in any other research studies?  

Yes  No  

Have you taken part in any other research study in the last 3 months?  

Yes  No  

Section B: To be completed by the researcher  

1. Measurements  

Weight ........................................ Height........................................ BMI ........................................ (kg/m²)  

% Body Fat ...............................  

Blood pressure: R1 ............................... R2 ............................... R3 ...............................  

2. Consent  

Date informed consent obtained:  

..........................................................
B.3 Consent form

REG Number: 
Patient Identification Number for this trial: 
Study Number: 

CONSENT FORM

Title of study: Epigenetic regulation of Omega-3 polyunsaturated fatty acid synthesis 
(The ‘E-PUFA’ study)

Please initial box

☐ I have read and understood the information sheet (Version 5) dated 30th March 2015 for the above study. I have had the opportunity to ask questions and have had them answered satisfactorily.

☐ I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected and that I can contact the research team to tell them whether they can still use data/samples already collected from me.

☐ I agree for any unused blood samples to be used for further research related to this project (not DNA samples).

☐ I give consent to my GP being informed of my participation in this research study.

☐ I understand that data from the study may be looked at by regulatory authorities or by persons from the Trust where it is relevant to my taking part in this research. I give permission for these individuals to have access to this information.

☐ I understand that all personal data relating to volunteers is held and processed in the strictest confidence, and in accordance with the Data Protection Act (1998).

☐ I agree to being sent a copy of my blood test and blood pressure results.

☐ I agree to take part in the above study.

Name of volunteer (BLOCK CAPITALS)

.................................................................

Signed ........................................ Date .....................................................

Name of researcher/person taking consent (BLOCK CAPITALS)

.................................................................

Signed ........................................ Date .....................................................