Interaction of omega-3 fatty acids and cytokine gene DNA methylation

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Doctoral Thesis

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Thesis Summary

It is well established that inflammation is associated with many non-communicable diseases and that when there is a failure in the resolution process acute inflammation becomes chronic with many undesired consequences. Treatment of chronic inflammation with pharmacological anti-inflammatory drugs can have detrimental effects on the body and can lead to an immunocompromised state. It is therefore prudent to consider whether other interventions can combat chronic inflammation.

It is possible that omega-3 polyunsaturated fatty acids (ω-3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), high levels of which are found in fatty fish, can influence the inflammatory process in a beneficial way and promote the resolution of inflammation thus facilitating a return to homeostasis. The mechanisms through which this might be achieved are not fully understood but the answer may lie within the epigenetic changes that are provoked by the presence of the fatty acids in the diet.

Epigenetics is a rapidly developing field of study which investigates heritable chemical modifications to the genome which are independent of the DNA sequence. These chemical changes are vital for normal cellular development, cellular processes, and cell-specific gene expression profiles; and if ω-3 PUFAs (EPA and DHA) could influence them, they could provide a target for therapeutic interventions.

Research to date has demonstrated that ω-3 PUFA (EPA and DHA) supplementation is associated with changes to markers of inflammation and changes in gene expression. Few studies have researched the effect of dietary ω-3 PUFA (EPA and DHA) supplementation on the epigenome and none have focused specifically on the inflammatory pathways associated with chronic inflammation in adult humans. The doctoral research reported in this thesis aims to elucidate the interaction between dietary fatty acids and the epigenome particularly as regards inflammation and its resolution.

Focusing on one epigenetic marker, DNA methylation, this work has employed a hypothesis driven targeted approach with candidate genes selected after a review of relevant literature. Custom designed pyrosequencing assays were developed to

facilitate the measurement of DNA methylation at specific cytosine bases in identified biologically relevant stretches of DNA.

We performed a cross sectional study to investigate the relationship between methylation levels of the gene which encodes tumor necrosis factor alpha (TNFα), and levels of fatty acids within the blood. It was found that many of the fatty acids included in the study were not correlated with methylation levels of *TNF* but some significant correlations were discovered. By analysing the results by sex it was also discovered that some correlations are sex-specific. Specifically, for the males within the study, significant correlations were observed between *TNF* methylation and ω-3 PUFA DHA, as well as ω-6 PUFA arachidonic acid (AA).

The relationship between blood fatty acid levels and DNA methylation of cytokine genes was further investigated using a four-week ω-3 PUFA (EPA and DHA) supplementation intervention. The genes included in this study were *TNF*, interleukin 6 (*IL6*) and interleukin 1 beta (*IL1B*). Peripheral blood mononuclear cells were isolated from blood samples before and after supplementation and these were analysed for fatty acid levels in the cell membranes in order to explore the timeframe over which incorporation occurred. Despite seeing an increase in ω-3 PUFAs EPA and DHA in PBMCs, there were no changes in methylation of the candidate genes over the intervention period and therefore postulated that this might be because an inflammatory stimulus was absent in this cohort of healthy males.

In the final study of this thesis methylation of candidate genes at biologically relevant sites cytokines and RNA expression were measured before and after an inflammatory stimuli, and either side of fatty acid supplementation. In this randomised double blind, repeated measures supplementation study the inflammation stimulus was provided by eccentric exercise and one group was supplemented with ω-3 PUFA (EPA and DHA) and the other with olive oil. Baseline measurements, made before the inflammatory stimulus or supplementation, showed that methylation levels of *TNF* were negatively associated with the expression of *TNF* mRNA, however there was no association with TNFα cytokine levels. There was a negative relationship between *TNF* and *IL1B* methylation levels at baseline.

The inflammatory stimulus induced a decrease in *TNF* and *IL6* methylation levels and an increase of serum cytokines coded by these genes. In contrast, an increase in methylation in the measured region of *IL1B* was observed. The results clearly demonstrated the effect of a repeated bout of exercise in which decreased inflammation (cytokines and muscle damage markers) is observed after the second bout. There was no difference in the decrease as a consequence of the dietary supplementation.

In conclusion it was found that as a result of the cross-sectional study, that some but not all fatty acids in the blood affect the methylation levels of certain CpG sites in exon1 of *TNF.* Positive and negative associations were observed, with many of the associations being sex specific. In the second study it was established that a four-week period of dietary ω-3 PUFA (EPA and DHA) supplementation does not significantly change the methylation levels of *TNF*, *IL6* or *IL1B* and, in the final study, muscle damage was induced through eccentric exercise and this effected a transient epigenetic change, but that fatty acid supplementation had no significant effect over the period of the study. Although DNA methylation of *TNF*, *IL6* and *IL1B* at the sites studied is not altered by a change in blood fatty acids over a short period this may not be the case over longer periods of intervention and this, together with exploration of the interaction with other environmental factors, such as muscle induced inflammatory stimulus, present opportunities for future research.

Acknowledgements

When I was young, in an interview for a local newspaper, I was asked what I wanted to be when I grew up. Then, just a little girl, I responded that I wanted more than anything else to be 'A Scientist'. I am so proud to say, I have made my childhood dreams come true. None of that would have been possible without all the support, time, encouragement and love from those around me. I would like to thank you all, I am greatly indebted to you.

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Abbreviations

Abbreviations	Descriptions
%TFA	percentages of total fatty acid
450k array	Illumina Infinium HumanMethylation450 BeadChip Arrays™
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AA	w-6 PUFA arachidonic acid
ALA	alpha-linolenic acid
ANOVA	Analysis of variance
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
BIA	Bioelectrical impedance analysis
BMI	Body Mass Index
bp	base pairs
BS-WGS	bisulphite whole genome sequencing
BV	blood volume
BWW	Between, Within, Within
CBA	Cytometric Bead Array
CBMCs	cord blood mononuclear cells
CCL17	chemokine ligand 17
CD14	cluster of differentiation 14 gene
CD14	cluster of differentiation 14
CD36	cluster of differentiation 36 gene
CD36	cluster of differentiation 36
cDNA	complementary DNA
CDS	Coding DNA Sequence
CHD	chronic heart disease
CK	Creatin kinase
COPD	chronic obstructive pulmonary disease
CpG	Cytosine-Guanine dinucleotides
CRP	C-reactive protein
CV	red cell volume
CVD	Cardiovascular disease
DBP	diastolic blood pressure
DBS	dried blood spots
DGLA	Dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DMRs	differentially methylated regions
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DPA	docosapentaenoic acid

EDTA	Ethylenediaminetetraacetic acid
EEIMD	eccentric exercise induced muscle damage
ELISAs	enzyme-linked immunosorbent assays
ELOVL	Elongation Of Very Long Chain Fatty Acids Protein
ELOVL	ELOVL Fatty Acid Elongase gene
EMBL-EBI	European Molecular Biology Laboratory - European Bioinformatics Institute
EPA	Eicosapentaenoic acid
EVOO	extra virgin olive oil
EWAS	Epigenome wide associations studies
FACS	fluorescence-activated cell sorting
FADS1	Fatty Acid Desaturase 1 gene
FADS2	Fatty Acid Desaturase 2 gene
FAMEs	fatty acid methyl esters
FAS	fas cell surface death receptor
FFAR3	Free Fatty Acid Receptor 3
FFQ	Food frequency questionnaire
FOXP3	Forkhead Box P3
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA3	GATA binding protein 3 gene
GC-MS	Gas chromatography mass spectrometry
GLA	γ-Linolenic acid
GPR120	G-coupled protein receptor 120
HAT	histone acetyltransferase
Hb	haemoglobin
Hct	haematocrit
HDAC	histone deacetylase
HDL	high density lipoproteins
HLTF	helicase like transcription factor
HPLC	high performance liquid chromatography
hs-CRP	high sensitivity C-reactive protein
IFNG	interferon gamma
IL	interleukin
IPAQ	International Physical Activity Questionnaire
LA	linoleic acid
LDH	lactate dehydrogenase
LDL	low density lipoproteins
LEP	leptin
LEPR	leptin receptor
LINEs	long interspersed nucleotide elements
LPS	lipopolysaccharide
LUMA	luminometric methylation assay
Mb	myoglobin
MEM	Modified Eagle Media

meth-GWAS	Methylation - genome-wide association studies
methylation	methylation of cytosine
MetS	metabolic syndrome
MMP13	Matrix Metallopeptidase 13
mRNA	messenger RNA
MSP	methylation-sensitive PCR
MUFA	Monounsaturated fatty acid
NCBI	National Centre for Biotechnology Information
NFκB	nuclear factor kappa B
NOS2	nitric oxide synthase 2
noseq	no sequencing primer
NSAID's	nonsteroidal anti-inflammatory drugs
NTC	no template control
OA	Oleic acid
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCG-1*α*	PPAR coactivator 1 alpha
PCR	polymerase chain reaction
PDK4	pyruvate dehydrogenase kinase 4
PMA	phorbol myristate acetate
POMC	pro-opiomelanocortin
Post-EE	Immediately after undertaking eccentric exercise
Post-EE+0.5h	30 minutes after undertaking eccentric exercise
Post-EE+1.5h	One and a half hours after undertaking eccentric exercise
Post-EE+3h	Three hours after undertaking eccentric exercise
Post-EE+48h	48 hours after undertaking eccentric exercise
Post-PT1	After undertaking performance test 1
Post-PT2	After undertaking performance test 2
Post-PT3	After undertaking performance test
Post-PT4	After undertaking performance test 4
PPAR	peroxisome proliferator-activated receptor
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
PPARG	the gene peroxisome proliferator-activated receptor gamma
PPARGC1A	PPAR coactivator 1 alpha (PGC1alpha)" gene
Pre-EE	Baseline measurements before eccentric exercise
Pre-PT3	Before undertaking performance test 3
Pre-PT4	Before undertaking performance test 4
PUFA	Polyunsaturated fatty acid
PV	plasma volume
Qiagen	Qiagen N.V. and its subsidiaries
RBC	red blood cells
RCT	randomised control trial

RNA	ribonucleic acid
ROM	range of motion
SAM	S-adenyl methionine
SFA	Saturated fatty acid
SNPs	single nucleotide polymorphisms
SPM	Specialised pro-resolving mediators
STAT3	signal transducer and activator of transcription
T2DM	type two diabetes mellitus
TET	Ten-eleven translocation
TFAM	transcription factor A mitochondrial
TG	Triglyceride
TLR4	toll like receptor 4
TNF	Tumor Necrosis Factor
TNFα	Tumor Necrosis Factor alpha
TRIAL	experimental trial days
tRNA	transfer ribonucleic acid
TSS	Transcription Start Site
uncon	unmethylated non-converted DNA
VAS	visual analogue scale
WBC	white blood cells
ω-3	Omega 3
ω-6	Omega 6

List of Publications

Publications

Hussey B., Lindley MR., & Mastana SS. (2017) Epigenetics and Epigenomics: the future of nutritional interventions? Future Sci. OA FSO237

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Hunter DJ., Wilson L., **Hussey B.**, Wadley AJ., Lindley MR., & Mastana SS. (SUBMITTED) Impact of aerobic exercise and fatty acid supplementation on global and gene-specific DNA methylation.

Wadley AJ., Keane G., Cullen T., Wilson L., Vautrinot J., Davies M., **Hussey B.**, Hunter DJ., Mastana S., Holliday A., Petersen S., Bishop NC., Lindley MR., & Coles SJ., (SUBMITTED) Characterisation of extracellular antioxidant enzyme concentrations in response to exercise in humans. *Submitted & currently under review.*

Conference Proceedings

Hussey B., Hunter D.J., Wilson L., Lindley M.R., & Mastana S.S. DNA methylation of *TNF* decreases after an intense bout of eccentric exercise. (Abstract #1642). Presented at the 67th Annual Meeting of The American Society of Human Genetics, October 2017, Orlando, FL.

Steel R., **Hussey B.,** Mastana S.S., Lindley M.R., & Taylor I.M. (2018) The associations among motivation, health-related behaviours, and the DNA methylation of TNFα. (Abstract #655). Presented at the 32nd Annual Conference of the European Health Psychology Society,

Hunter D.J., **Hussey B.**, Wilson L., Lindley M.R., & Mastana S.S. DNA methylation of *PPARGC1A* is associated with cycling performance. (Abstract #1658). Presented at the 67th Annual Meeting of The American Society of Human Genetics, October 2017, Orlando, FL.

Chapter 1 Introduction

1.1 The Human Genome Project and Epigenetics

For many years it was commonly accepted that sequencing the human genome would establish the origins of numerous diseases and genetic disorders as well as providing a means for curing them. However this perception was not realised, because after years of research and billions of dollars of funding, the Human Genome Project provided the script for the human genome but not the great leap in understanding that was sometimes anticipated (Wade, 2010; Non and Thayer, 2015). This has led to there being many more questions than answers and the key to answering them may lie in the rapidly expanding fields of epigenetics and epigenomics.

Epigenetics as a term was introduced in the 1940's by Conrad Waddington (Waddington, 1942, reprinted in 2012). It originally referred to the study of how cells with the same genetic code could differentiate into the vast array of different cell types within an organism, connecting the genotype with the phenotype. Over time usage of the term has changed and today the field of epigenetics, which literally means "above genetics", investigates chemical changes to DNA that do not alter the gene sequence itself (Holliday, 2006; Bernstein, Meissner and Lander, 2007; Huang, Jiang and Zhang, 2014). Epigenetics focuses upon how and when chemical modifications alter a specific gene's expression, whilst epigenomics considers the overall effect of many epigenetic changes throughout the whole genome (Fazzari and Greally, 2004).

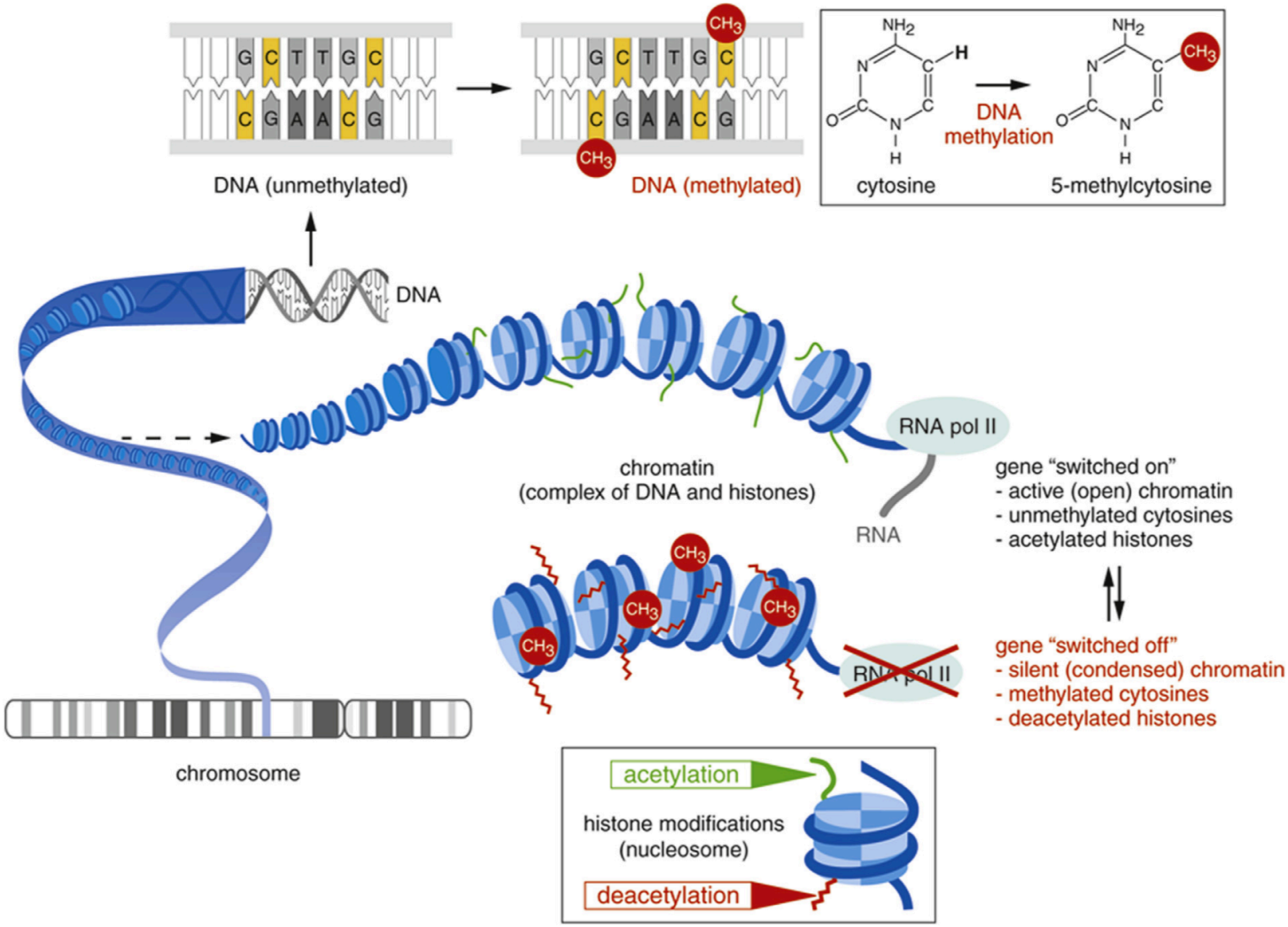

Figure 1-1 Epigenetic chromatin modifications, reproduced with permission from Mukherjee, Twyman and Vilcinskas, (2015)

The chemical changes with which epigenetics concerns itself are illustrated in Figure 1-1 and include DNA methylation, histone modifications, chromatin remodelling and non-coding ribonucleic acids (RNAs; Jenuwein, 2001; Bird, 2002; Goll and Bestor, 2005; Mattick and Makunin, 2006; Barski *et al.*, 2007; Berger, 2007; Kouzarides, 2007). These epigenetic changes are vital for, and part of, normal cellular development, cellular processes, and cell-specific gene expression profiles (Bernstein, Meissner and Lander, 2007; Jones and Baylin, 2007). Epigenetic modifications of the DNA alter regulate transcription through changes in the accessibility of the gene's transcriptional regions, such as the promotor (Liu, Jin and Zhou, 2015). The modifications alter the chromatin structure to either be open and accessible, or closed and condensed. The ability of a transcription factor to bind to a particular gene promotor is the culmination of epigenetic events including histone modifications, nucleosome position, nuclear localisation and DNA methylation status which allow for accessible chromatin (Liu, Jin and Zhou, 2015).

Epigenetic modifications are often stable in adults and can pass from one generation to another (Reik, 2007), giving rise to the concept of epigenetic memory (D'Urso and Brickner, 2014). This epigenetic stability helps maintain cell identity and provides genomic integrity. However, epigenetic responses are not always stable; sometimes they are short-lived and are reversed allowing the cell to adapt to the environment (Ivashkiv, 2013). The majority of epigenetic modifications occur during development (Santos and Dean, 2004), during this period it is essential for mammalian development (Smith and Meissner, 2013). After fertilisation of the egg, the DNA methylation of the paternal genome after fertilisation is rapidly demethylated, followed by a passive demethylated of the maternal genome, this occurs prior to the methylation being re-written in order to assist in cell-linage decisions (Smallwood and Kelsey, 2012). There are exceptions of these epigenetic changes during development, which do not demonstrate the same demethylation, these are imprinted genes; these genes are expressed from either the maternal or paternal allele and maintain their DNA methylation status during the epigenetic reprogramming of early development.

Through epigenetic modifications of the genome allow the cell to adapt and respond to the environment, however sometimes the changes are not beneficial, and such changes, together with errors in epigenetic modifications can result in disease (Portela

and Esteller, 2010; Park, Friso and Choi, 2012; Brunet and Berger, 2014; Benayoun, Pollina and Brunet, 2015). In such cases an understanding of the aberrant epigenetic changes and its causes can provide a target for therapeutic interventions (Lu *et al.*, 2006; Kelly, De Carvalho and Jones, 2010).

Much of the current understanding of the regulation of gene expression comes from studies of DNA methylation, which is an important epigenetic marker for regulation of gene expression (Morgan *et al.*, 2017). It has been shown that gene methylation levels are implicated in disease and inflammation (Jin and Liu, 2018). Measurement of methylation levels is relatively easy and small changes to status over relatively short sections of DNA can have a big impact upon gene expression (Piperi *et al.*, 2008). The fact that DNA methylation is such a widespread epigenetic modification, the impact varying levels of methylation have on gene expression and the ability to accurately measure those levels, combine to make methylation analysis such an important research tool and it is the one that is utilised in this research.

In recent years there has been an exponential increase in epigenetic research and knowledge, and as a consequence the interactions between diverse environmental factors are beginning to be pieced together, the genome and the epigenome to provide some understanding of the potential implications for health and disease (Bollati and Baccarelli, 2010; Muka *et al.*, 2016). One such key environmental factor to which humans are exposed from pre-conception to death is nutrition, which before birth is provided by the mother, and afterwards by diet.

1.2 The diet and nutri-epigenomics

Nutri-epigenomics seeks to delineate the interactions between the diet and the genome through epigenetic mechanisms (Anderson, Sant and Dolinoy, 2012; Zhang, 2015). The ability of the epigenome to adapt to environmental factors, including diet, is referred to as “plasticity” and this alters across the lifespan of an organism (Gallou-Kabani *et al.*, 2007). Thus, there are periods during which the epigenome is more responsive to change (Kanherkar, Bhatia-dey and Csoka, 2014), for example increased plasticity occurs in pre-natal and neonatal phases (Reik, 2007; Foley *et al.*, 2008), when cell differentiation and specialisation is taking place. Environmental

factors, such as nutrition, therefore have more influence during these periods of development (Gluckman *et al.*, 2008). Sometimes the epigenetic consequences can be quite dramatic, for example when genetically identical larvae of the honeybee are fed royal jelly, the DNA methylation patterns are altered, resulting in differentiation into a Queen bee rather than a worker bee (Kucharski *et al.*, 2008). In mammals, malnutrition of methyl donors vitamin B_{12} and choline during gestation can lead to epigenetic dysregulation within the offspring, giving rise to obesity and influencing disease in later life (Waterland and Jirtle, 2003).

The Developmental Origins of Health and Disease hypothesis explains how abnormalities within the developmental environment can affect the epigenome and lead to metabolic disease later in life (Gluckman and Hanson, 2004a). It is understood, for example, that maternal stress (Oberlander *et al.*, 2008), birth weight (Burdge, Lillycrop and Jackson, 2009) and foetal malnutrition (Steegers-Theunissen *et al.*, 2009) can all impact upon the developmental environment, and that the consequential modifications to the epigenome can endure throughout the lifespan and that some are even passed onto future generations (Burdge *et al.*, 2007). Key nutrients can protect against the detrimental effect of other environmental factors; for example, it has been shown that supplementation with omega-3 (ω-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) during gestation has a protective effect against demethylation caused by the negative behaviour of smoking during pregnancy (Lee *et al.*, 2013).

Since the epigenome is especially responsive to change during gestation, the provision of a favourable developmental environment, including maternal diet, should be beneficial to the wellbeing of offspring and future generations. However, if diet in early life has an epigenetic consequence then it is reasonable to conclude that diet in general, i.e. throughout a person's lifespan, could also have such consequences. This is an important consideration because aberrant alterations to epigenetic regulation are implicated in many chronic diseases including cancer, chronic obstructive pulmonary disease, cardiovascular disease (CVD), obesity, neurological disorders, and type 2 diabetes, to name a few (Portela and Esteller, 2010; Park, Friso and Choi, 2012; Brunet and Berger, 2014; Benayoun, Pollina and Brunet, 2015). If it can be determined whether, and to what extent, diet is involved in the epigenetic changes that are

associated with these diseases would it be possible to develop an “epigenetically healthy” diet to reduce their negative impact?

Convincing individuals to maintain an “epigenetically healthy diet” in order to benefit their long-term health and that of their children and grandchildren might not be easily achieved for many reasons, one of which is that long term lifestyle changes do not offer an individual an immediate beneficial effect. However the epigenetic changes brought about by royal jelly in the honeybee larvae are an example of when even short term changes to diet can have significant consequence, therefore research into the short-term effects of diet on the epigenome may present opportunities which individuals would find attractive and to which they may be persuaded to adopt, particularly if it could be demonstrated that they would personally benefit.

Personalised medicine and interventions are increasingly commonplace and seen as a way to both improve health care and lower costs (Vogenberg, Isaacson Barash and Pursel, 2010). For example, pharmacogenomics uses the genome of an individual in order to establish if they are responders or non-responders to particular drugs and to establish what dosage is appropriate (NIH, 2018). There has also been an increase in the use of genetic testing to identify targeted populations and interventions that will reduce the burden of disease in later life (The Academy of Medical Sciences, 2015). Consequently, there is building evidence to suggest that epigenetic signatures could also be used to personalise interventions and predict response status (Campión *et al.*, 2009).

As research reveals more information about changes in the epigenome, it provides new therapeutic targets for interventions. Drugs have been developed to target epigenetic regulating enzymes, such as DNA methyltransferases (DNMT), histone deacetylase (HDAC) and histone acetyltransferase (HAT) inhibitors in order to reverse the changes to the epigenome. There is huge potential for epigenetic drugs, as discussed in *Clinical Epigenetics* (Altucci and Rots, 2016), however, there is a long way to go before the impact of such drugs is truly understood, particularly as regards the wider alterations to the epigenome. Perhaps nutritional interventions or dietary supplementation could provide a safer and more cost-effective approach to prevent or

reset aberrant epigenetic changes by influencing the natural epigenetic programming within cells.

Whilst nutritional interventions may be seen as less invasive and safer than 'epigenetic drugs', it is still important to understand how they work and whether they really do provide the benefits that are frequently suggested. This means that interactions at a molecular level need to be fully understood so that their full consequences can be understood, and future research can determine how a rewarding benefit for public health can be achieved.

1.3 Scope of this research

There has been increasing evidence on the health benefits of fish oil supplements. The 'active ingredients' of fish oil supplements are the ω-3 fatty acids EPA and DHA (Calder, 2008, 2012a). This thesis aims to consider the interactions between ω-3 fatty acids and DNA methylation in order to understand how ω-3 fatty acids alter cytokine gene expression and provide a greater understanding of fatty acid involvement in inflammation.

Chapter 2 Background and Literature Review

2.1 What is inflammation?

Inflammation has been recognised since the 1st century AD when Cornelius Celsus documented four main signs associated with it; redness, swelling, heat and pain (Rocha e Silva, 1978). Since the middle of the 20th century, there has been increased research into the inflammatory process leading to our current understanding that these clinical signs are the manifestation of a complex cascade of events which can be considered at molecular, cellular and physiological levels (Scott, 2004).

This research is concerned with acute inflammation arising from induced damage to muscles and whether the inflammatory response can be influenced by the dietary intake of ω-3 PUFAs. It is therefore appropriate to discuss the process of inflammation at molecular and cellular levels since this informs the decisions made here regarding measurement of the induced inflammatory response. Thus, the following explanation seeks to provide background to the type of inflammatory response that this study is concerned with and does not consider inflammation mechanisms specific to immune related disorders.

Acute inflammation is the initial response to various types of trauma, one of which is mechanical loading (Scott, 2004). The inflammatory response is mediated and regulated by the interactions of many cell types for example monocytes, macrophages and lymphocytes. During an acute inflammatory response, chemical mediators are released from activated cells and trigger the inflammatory response, these chemical mediators include cytokines, chemokines, eicosanoids and reactive oxygen species. As an example, monocytes within the circulation are surveillance and antigen presenting cells which give rise to macrophages when entering tissue. When responding to a stimulus, activated monocytes and macrophages are a source of eicosanoids, and a variety of cytokines including IL6, TNFα and IL1β. These proinflammatory cytokines and chemokines activate macrophages and neutrophils which in turn activate leukocytes to infiltrate the site of the trauma and attract neutrophils to the site through a chemotaxis gradient.

The acute inflammatory response is self-regulating through the activation of negative feedback mechanisms. However, when this tightly controlled process becomes unregulated this can result in chronic, systemic inflammation characterised by activated inflammatory cells and elevated inflammatory markers. There are increased concentrations of inflammatory cytokines such as IL6, TNFα and IL1β, which can cause damage to tissues throughout the body. Chronic diseases such as rheumatoid arthritis and inflammatory bowel disease are key examples of when this happens.

Fatty acids play key roles within the inflammatory process. These roles include direct action through fatty acid receptors on the cell surface and within the cell, incorporation into the cell membrane and as precursors to other molecules including eicosanoids and prostaglandins. The interactions between fatty acids and the inflammatory process are discussed in more detail below, however first we must understand what fatty acids are.

2.2 What are fatty acids?

Whilst a full consideration of what fatty acids are, their properties and chemistry is beyond the scope of this thesis a basic understanding of what these molecules are will help put this research in context.

Fatty acids are amphipathic small molecules which are formed by a hydrocarbon chain that has a methyl group at one end and a carboxylic acid group at the other (Fahy *et al.*, 2005; Rustan and Drevon, 2005). Alterations in the length and structure of the hydrocarbon chain results in the different types of fatty acids which have varying properties (Gunstone, 1996).

When the hydrocarbon chain ("backbone") of a fatty acid does not contain any double bonds the carbon atoms are fully saturated with hydrogen it is known as a saturated fatty acid (SFA). Saturated fatty acids have the general formula $CH_3(CH_2)_nCOOH$. For example, steric acid (also known as Octadecanoic acid) is a long chain SFA found as a major component in cocoa butter and has the formula $CH_3(CH_2)_{16}COOH$, illustrated in Figure 2-1.

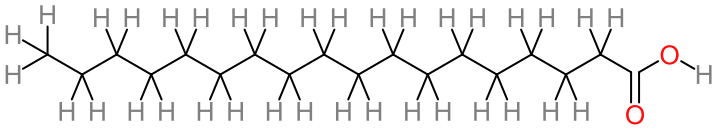

Figure 2-1 Chemical structure of Stearic acid, a saturated fatty acid (SFA)

SFAs are relatively stable, have a lower reactivity, and their melting points tend to be higher than the more complicated fatty acids discussed below which include one or more double bonds in the carbon backbone. See Table 2-1. They are therefore relatively inert, and those with longer carbon backbones are oxidised at a slower rate than those with shorter chains (Leyton, Drury and Crawford, 1987). For example lauric acid, a 12 carbon saturated fatty acid more highly oxidised than the 18 carbon long stearic acid (DeLany *et al.*, 2000).

Table 2-1 Melting points of saturated fatty acids, data from Lide (2007)

	Number of carbons	Melting point (°C)
13:0 (Tridecanoic acid)	13	41.5
14:0 (Myristic acid)	14	54.2
15:0 (Pentadecanoic acid)	15	52.3
16:0 (Palmitic acid)	16	62.5
18:0 (Stearic acid)	18	69.3
20:0 (Arachidic acid)	20	76.5
21:0 (Heneicosylic acid)	21	73.5
22:0 (Behenic acid)	22	81.5

When the hydrocarbon backbone includes a single double bond, a kink in the chain is formed. A fatty acid which includes such a double bond is known as a monounsaturated fatty acid (MUFA). Monounsaturated fatty acids have the general formula $CH_3(CH_2)_n–CH=CH–(CH_2)_n–COOH$. The length of the carbon chain and the position of the double bond vary between different MUFAs. There are two orientations of the functional groups around the double bond. When the functional groups are on the same side of the double bond the molecule has a *cis* formation, and when they are on opposite sides the molecule has a trans formation. Oleic acid (also known as Cis-9-Octadecenoic acid) is a MUFA having the formula $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$, illustrated in Figure 2-2. The double bond causes MUFAs to have a lower melting point than SFAs, which is further reduced when the double bond is in the *cis* formation as opposed to the *trans* form (Gunstone, 1996).

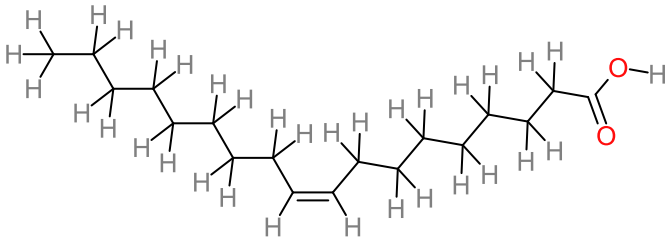

Figure 2-2 Chemical structure of Oleic acid, an omega-9 monounsaturated fatty acid (ω-9 MUFA)

The addition of further double bonds to the hydrocarbon backbone results in polyunsaturated fatty acids (PUFAs). The location of the first double bond from the methyl end of the chain in both MUFAs and PUFAs leads to their designation by omega (ω) number. Naturally occurring fatty acids are generally in the cis orientation and typically the first double bond is positioned at the third (ω-3), sixth (ω-6) or ninth (ω-9) carbon from the methyl end of the hydrocarbon chain (Ratnayake and Galli, 2009). The ω-3 PUFA EPA and the ω-3 PUFA DHA, both of which are constituents of fish oil dietary supplements, are illustrated in Figure 2-3. Eicosapentaenoic acid has a 20-carbon atom chain and 5 cis double bonds. Docosahexaenoic acid has a 22-carbon atom chain and 6 cis double bonds.

A) B)

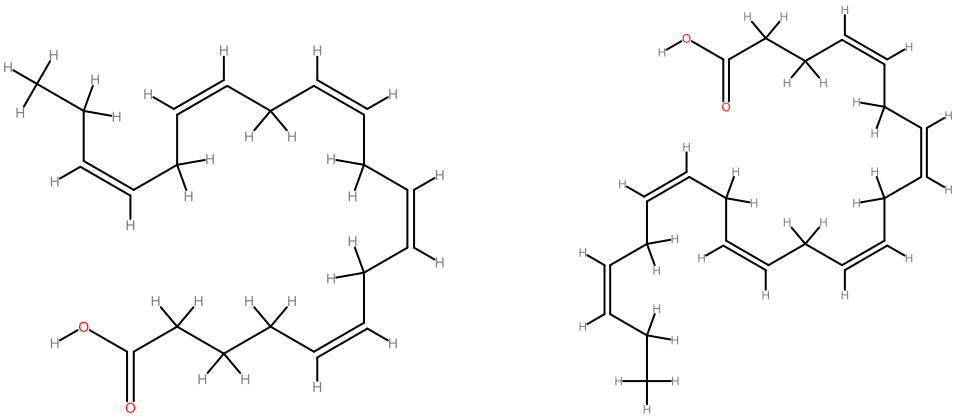

Figure 2-3 Chemical structure of A) eicosapentaenoic acid (EPA) and B) docosahexaenoic acid (DHA), omega-3 polyunsaturated fatty acids (ω-3 PUFA) found in fish oil supplements.

2.2.1 Biological significance of fatty acids: focus on PUFAs

Fatty acids play key roles within the body, including: energy transport and storage, components of cell membranes, thermal, electrical and mechanical insulation, and assisting in cell signalling (e.g. lipid mediators and gene regulation; Arab, 2003; Rustan and Drevon, 2005; Ratnayake and Galli, 2009).

Many of the biologically important fatty acids can be synthesised within the body but this is not universally true, and some must form part of an individual's diet, and are designated as essential dietary fatty acids. Dietary fatty acids were first found to be essential to health in the late 1920's when it was demonstrated that feeding a rat a fat-deficient diet led to disease and ultimately to their death. However the addition of a few drops of fat to a diseased rats diet returned it to health (Burr and Burr, 1929). Further research by Burr & Burr (1930) demonstrated that it was two unsaturated fatty acids, ω-6 PUFA linoleic acid (LA) and ω-3 PUFA alpha-linolenic acid (ALA), that are essential to mammals in their diet. It transpires that mammals are unable to synthesise these two fatty acids because they lack the enzymes 12-desaturase and 15-desaturase required to desaturate fatty acids at the ω-3 and the ω-6 positions, whereas plants do have these enzymes (Calder, 2004). Therefore, mammals rely on these essential fatty acids to have entered the food chain from plants. The ω-6 PUFA LA is in fact the most abundant of the PUFAs within the human diet (Calder and Grimble, 2002), and is found in vegetable oils including, for example sunflower oil, corn oil and soybean oil. Comparatively, dietary ω-3 PUFA ALA, which is found for example in flaxseed, soybeans and walnuts, is 5-10 fold less abundant (Mozaffarian, 2012).

The relative abundance, i.e. the ratio of ω-6 LA:ω-3 ALA, in the diet is of interest because this research is concerned with genes associated with the inflammatory response. Omega-3 fatty acids have been shown to be associated with an anti-inflammatory (pro-resolving) response, whilst ω-6 fatty acids have been shown to be associated with an inflammatory response (pro-inflammatory; Calder, 2012). It is also worth noting at this point that human beings evolved on a diet containing a ω-6:ω-3 ratio of approximately 1:1 (Simopoulos, 2006), whereas today's western diet contains a ratio of LA:ALA that varies across Europe ranging from 1:4.3 in females from

Denmark to 1:28.8 in males from the Netherlands (Burdge and Calder, 2005). Thus, human beings evolved with a diet in which pro-inflammatory and pro-resolving fatty acids were in balance, but humans now consume a diet in which pro-inflammatory ω-6 fatty acids predominate (Simopoulos, 2009). The human diet has therefore changed remarkably from that consumed over the evolutionary period and now there is also considerable variation in the consumption of essential fatty acids according to the geographic location of the consumer.

The dietary importance of the essential fatty acids LA and ALA arises because they are precursors to longer highly unsaturated and biologically relevant PUFAs, their metabolism is depicted in Figure 2-4. Firstly, the ω-6 PUFA arachidonic acid (AA) and the ω-3 PUFA eicosapentaenoic acid (EPA) are synthesised from LA and ALA respectively. EPA is further elongated to form DPA and then further desaturated to form DHA.

Although EPA and DHA are not essential fatty acids in the diet because the body can convert ALA to them, the conversion process is poor and the major source of EPA and DHA comes from the diet (Plourde and Cunnane, 2007). The inability of the body to efficiently synthesis these “conditionally” essential fatty acids and the consequential reliance on dietary intake, means that dietary choices can alter the availability of these acids in the body (Plourde and Cunnane, 2007).

Elongation of the fatty acid carbon chain is achieved by fatty acid enlongases and desaturation with the insertion of double bonds by Δ6-desaturase and Δ5-desaturase (Benatti *et al.*, 2004). The Δ6-desaturase and Δ5-desaturase enzymes are encoded by the genes *FADS2* “fatty acid desaturase 2” and *FADS1* “fatty acid desaturase 1” respectively. The steps in the fatty acid metabolic pathway that these enzymes catalyse are rate limiting (Marquardt *et al.*, 2000; Nakamura and Nara, 2004). In addition, both *in vivo* and *in vitro* studies have shown that LA and ALA compete within the same pathway for the Δ6-desaturase, with the ω-3 PUFA ALA suppressing the ω-6-PUFA metabolism (Mohrhauer *et al.*, 1967; Holman, 1998; Jump and Clarke, 1999). The endogenous process of desaturation and elongation of the ω-6 and ω-3 PUFAs occurs predominantly in the liver, but also in other tissues (Hughes and Dhiman, 2002).

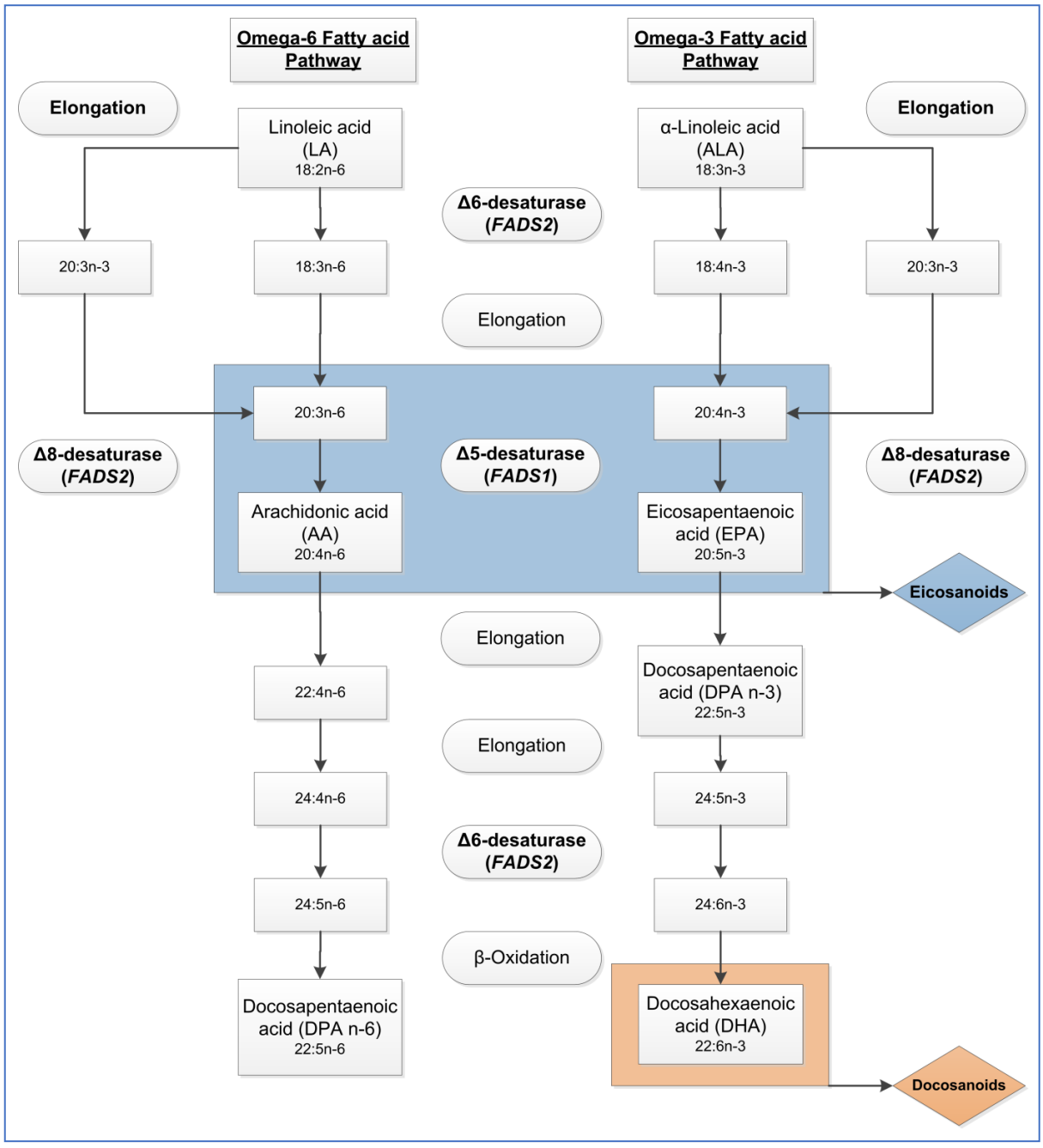

Figure 2-4 Omega 3 and Omega 6 Polyunsaturated Fatty Acid metabolism. The Δ6-desaturase and Δ5-desaturase enzymes are encoded by the genes FADS2 "fatty acid desaturase 2" and FADS1 "fatty acid desaturase 1". Eicosanoids and Docosanoids are key mediators and regulators of inflammation. Figure adapted from Glaser, Heinrich and Koletzko (2010).

Prior to the ground-breaking work of Burr and Burr in the early 20th Century, it was thought that fatty acids were just a source of calorific energy and thus interchangeable with carbohydrates (Smith and Mukhopadhyay, 2012). Whilst it is true that fatty acids do provide an important source of energy, which can be stored in adipose tissue and skeletal muscle, and which can be transported around the body in the blood, it is now known that they also have important and sometimes complex roles (Rustan and Drevon, 2005). The work carried out by the Burrs' demonstrated that linolenic acid played a physiological role in rats (Burr and Burr, 1929, 1930) and later work, such as that of Holman, demonstrated that it also plays a physiological role in humans (Holman, Johnson and Hatch, 1982). Indeed, the work of Holman showed that in humans a deficiency of linolenic acid had wide ranging debilitating effects, including numbness, paresthesia, weakness, inability to walk, pain in the legs and blurring of vision.

The wide-ranging effects observed by Holman reflect the varied and complex roles that fatty acids play in the body. Over time the biological significance of fatty acids and the way in which they act has been the subject of a considerable amount of research. For example, the skin abnormalities observed in the rats studied by the Burr's have been the subject of further research and the mechanisms for these observations have now been proposed; one example is the enzymic oxygenation of ceramides which aid in the function of the epidermal barrier (Zheng *et al.*, 2011). Fatty acids have also been found to influence diseases, sa demonstrated by the long chain ω-3 PUFA's influence on susecptibilty to cardiovascular disease through the beneficial influence of a range of risk factors associated with atherosclerosis, including a reduced production of inflammatory eicosanoids (Calder, 2004). Further evidence includes the cytoprotective capacity of long chain ω-3 PUFA's contribute to anti-angiogenic and neuroprotective mechanisms within the eye (SanGiovanni and Chew, 2005). The pro-physiological effect of ω-3 fatty acids has also been studied in relation to cancer and chronic illness (Colomer *et al.*, 2007). However, the physiological importance of fatty acids does not just extend to beneficial instances, since, for example, chronically elevated levels of plasma free fatty acids have adverse consequences and have been linked with the onset of peripheral and hepatic insulin resistance (Oh *et al.*, 2018; Spiller, Blüher and Hoffmann, 2018).

Fatty acids are major structural components of cells membranes, which are primarily formed from glycerophospholipids (commonly referred to as phospholipids) such as diacylglycerol and phosphatidic acid. Phospholipids are composed of a glycerol head and fatty acid backbone (Ratnayake and Galli, 2009). It is the amphipathic nature of these phospholipids which allow them to form cell membranes. Furthermore, the physical nature of the membrane is altered by the type of fatty acid that is incorporated into it (Stubbs and Smith, 1984). For example, if the proportion of unsaturated fatty acid incorporated within the cell membrane is increased, the fluidity of the membrane is itself increased (Abbott *et al.*, 2012). The type of fatty acid incorporated in the membrane also affects the lipid-protein interactions and the function of the membrane (Singer and Nicolson, 1972). Fatty acids, such as ω-3 PUFAs alter the composition of the membrane by increasing clustering of lipids rafts, resulting in the formation of large raft domains (Turk and Chapkin, 2013). Large lipid rafts can suppress the cell activation by impairing cell signalling (Chapkin et al., 2008; Kim et al., 2008), ultimately affecting cell function through suppression of downstream pathways, for examples those involved in inflammation.

While dietary SFAs have very little influence on cell membrane SFA content, as shown in rats; increasing the amount of dietary ω-3 PUFAs in the diet has been shown to increase the amounts of both ω-3 PUFAs EPA and DHA in the membrane phospholipids of both rats and humans (Yaqoob *et al.*, 2000; Kew *et al.*, 2004; Abbott *et al.*, 2012). Assessment of the ω-3 PUFA incorporation into red blood cells (RBCs) (Al-Hilai *et al.*, 2013; Amarasekera *et al.*, 2014; Albert *et al.*, 2015; Lind *et al.*, 2015) or plasma (Bouwens *et al.*, 2009; Block *et al.*, 2013; Abdolahi *et al.*, 2014; Augustine *et al.*, 2014) are therefore used to assess dietary PUFA intake. Foods rich in ω-3 PUFAs, such as salmon, leads to increased incorporation and modification of the cell phospholipid membrane (Raatz *et al.*, 2016). Displacement of the ω-6 PUFA AA form the membrane by ω-3 PUFAs EPA and DHA (Calder, 2015), occurs in a dose-dependent response manner (Trebble *et al.*, 2003). The composition of the cell membrane is altered in different ways by EPA and DHA separately (Mason *et al.*, 2016) and incorporation into the membrane can be modulated by genetic factors, for example a variant in the FADS1 gene (Takkunen *et al.*, 2016).

Once in the cell membrane, EPA and DHA compete with AA to be metabolised to lipid derived mediators, by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P monooxygenases 450 (CYP450; Arita, 2012; Wang *et al.*, 2012). Compared to the eicosanoids derived from the ω-6 PUFA AA which are potent inflammatory mediators, those derived from the ω-3 PUFAs are more anti-inflammatory in nature. Genetic polymorphisms COX and LOX genes have been linked to cancer, Alzheimer disease and myocardial infarction (Zhang *et al.*, 2015; Mashima and Okuyama, 2015) and are linked to the activity of the lipid mediator pathways (Tyurina *et al.*, 2014).

It is now understood that many different fatty acids are involved in a whole range of physiological processes and that their consumption, metabolism and synthesis are critical to life (Rustan and Drevon, 2005). Their involvement is now known to extend well beyond the traditionally understood role of providing a source of energy and the roles of fatty acids are of significant throughout the body and influence both health and disease.

2.3 PUFAs, disease and inflammation

The composition of fats within the diet has previously been scrutinised for their health benefits (Calder, 2006; Ruxton *et al.*, 2007; Khorsan *et al.*, 2014; Elagizi *et al.*, 2018). Populations with a diet rich in ω-3 PUFAs EPA and DHA have been shown to have a lower incidence of chronic non-communicable diseases (Simopoulos, 1999). The Mediterranean Diet for example, high in PUFAs and MUFAs, has been demonstrated to have beneficial effects on individuals with cardiovascular disease (CVD) by reducing the associated risk factors in comparison to the levels associated with a low-fat diet (Estruch *et al.*, 2006).

Considerable research has been undertaken in an attempt to establish whether it is the dietary ω-6 LA:ω-3 ALA ratio that has an impact upon disease manifestation in humans. Simopoulos (2002) concluded that a lower ratio reduced the risk of many chronic diseases. However, a cross-sectional study completed as part of the Tehran lipid and glucose study in Iran concluded that there was no association between the dietary ω-6/ω-3 PUFA ratio and metabolic syndrome (MetS; Mirmiran *et al.*, 2012).

Other researchers have found that intake of ω-6 PUFAs did not influence the beneficial effect of increased ω-3 PUFAs intake on the risk of CVD and chronic heart disease (CHD; Mozaffarian *et al.*, 2005; Fontes *et al.*, 2015). Mirmiran *et al.* (2012) found an inverse association between both ω-3 PUFA ALA and ω-6 PUFAs with MetS, the highest consumptions having lowest prevalence. In this study, no significant associations were found however between ω-3 EPA or ω-3 DHA and MetS. However, dietary intake of the ω-6 and ω-3 PUFAs was assessed using a food-frequency questionnaire (FFQ). This approach to data collection is inherently flawed because they rely on subject recall and it is difficult to achieve quantifiably precise records and they are not suitable when seeking to establish epigenetic relationships in detail.

Evidence that ω-3 PUFAs, such as EPA and DHA, can modulate inflammation has come from *in vitro* work (Endres *et al.*, 1989; Trebble *et al.*, 2003; Verlengia *et al.*, 2004), animal models (Yaqoob and Calder, 1995; Al-Khalifa *et al.*, 2012; Olson *et al.*, 2013; Richard *et al.*, 2016) and human studies (Endres *et al.*, 1989; Tartibian, Maleki and Abbasi, 2011; Itariu *et al.*, 2012); although the latter evidence is weaker as a

consequence of more complex environmental factors. The mechanisms through which ω-3 PUFAs promote an anti-inflammatory environment within the body are multifaceted and complex (Calder, 2015), and are illustrated in Figure 2-5.

Inflammatory diseases are often characterised with chronic activation of NFkB pathway (Lawrence, 2009). In its inactive form, NFkB is stored in the cytoplasm covalently bonded to its inhibitory subunit IkB (Jacobs and Harrison, 1998). After activation, IkB is phosphorylated by IKKs and this allows for the translocation of NFkB to the nucleus (Israel, 2010). ω-3 PUFAs have been shown reduce activation of NFkB by multiple mechanisms. Ligand bound PPARy can interact with NFkB by preventing its translocation to the nucleus (Calder, 2012b). EPA has also been shown to inhibit the translocation of NFkB subunits to the nucleus directly. Zhao et al., (2004) showed THP-1 cells treated with EPA had decreased translocation of the p65 subunit to the nucleus following LPS stimulation; pre-stimulation with EPA did not alter cytoplasm p65 and p50 content. EPA treatment resulted in lower NFkB activity after LPS stimulation and reduced TNFα mRNA expression and cytokine production (Zhao *et al.*, 2004). EPA treatment also supressed the phosphorylation of IkB-a in the cytoplasm, indicating that degradation of IkB-a and translocation of NFkB to the nucleus may be prevented by EPA through decreased phosphorylation of IkBα. Reduced activation of NFkB by ω-3 PUFAs in response to inflammation ultimately results in reduced expression of its target genes, such as cytokines. Treatment in vivo with EPA and DHA can modulate the expression of multiple inflammatory genes within the NFkB pathway (Allam-Ndoul *et al.*, 2016). *In vitro* models have demonstrated that treatment with EPA and/or DHA reduces cytokine production after stimulation with LPS.

Treatment of cells ex vivo with an inflammatory stimulus, for example LPS, and measurement cytokine production, can be used to measure the effect of in vivo supplementation with ω-3 PUFAs on the inflammatory response. Decreased production of TNFα, TNFR2, IL6, IL-1b, IL2, IFNγ and CRP have been observed when cells are stimulated ex vivo after ω-3 PUFA supplementation (Verlengia *et al.*, 2004). Some studies have examined the effect of EPA and DHA separately, DHA is shown to have a greater inhibitory effect on TNFα, IL-1b IL6, (Weldon *et al.*, 2007). Gene expression for TNFα, IL1β and IL6 were measured and were consistent with the

cytokine data (Weldon *et al.*, 2007). Whereas, EPA decreases production of IL2 and IFNγ to a greater extent than DHA (Verlengia *et al.*, 2004). The dosage of supplementation has also been shown to have an effect; three doses of ω-3 PUFAs over 12 weeks in males resulted in a 'U-shaped' dose-response curve with maximum inhibitory effects TNFα and IL6 production at 1g/day compared to the 0.3 and 2g/day (Trebble *et al.*, 2003).

Modulation of inflammatory gene expression also occurs through transcription factors. Once the ω-3 PUFAs are incorporated in the cell membrane they can be released into the cell and can interact with these transcription factors, such as PPAR, in the regulation of gene expression. Activation of PPAR occurs when ligands, including ω-3 PUFAs and eicosanoids, are non-covalently bonded to them. PPARγ and PPARα are the most understood of the PPAR isoforms (Tyagi *et al.*, 2011). Of interest here, PPARγ is expressed in inflammatory cells and has an anti-inflammatory action. In a cancer cell line, treatment with EPA and DHA have shown to increase expression of PPARγ (Rovito *et al.*, 2013). DHA interacts with PPARγ resulting in reduced cytokine production after stimulation (Li *et al.*, 2005). PPARγ is also involved in regulating metabolism in adipocytes with downstream effects promoting insulin sensitivity and blood triglyceride concentrations (Poynter and Daynes, 1998; Dunning *et al.*, 2014; Chiazza and Collino, 2016). Expression of PPARγ is modulated by DNA methylation within its promotor (Fujiki et al., 2009). In a case-control study of T2DM patients, DNA methylation was significantly different in genes involved in inflammation, among other pathways; differential methylation was also shown in T2DM candidate genes, including *PPARG* (Nilsson et al., 2014).

Alterations to the fluidity of the cell membrane, through changes in fatty acid composition, has an impact on the function of proteins within the membrane, cell signalling and gene expression. Specific genes have been found to be differentially expressed by alterations in ω-3 PUFA levels. The membrane glycoprotein which promotes inflammation in monocytes and macrophages, CD36 “cluster of differentiation 36”, has been found to be differentially expressed as the result of ω-3 PUFA supplementation. CD36 is a membrane glycoprotein expressed on the surface of many cells that are active in fatty acid metabolism, such as adipocytes, muscle cells, platelets, monocytes, heart and intestine cells (Kuriki et al., 2002). The CD36 protein

functions include the uptake of long-chain PUFAs, regulation of angiogenesis, scavenger in innate immunity, and has a key role in foam cell formation (Febbraio, Hajjar and Silverstein, 2001). In human skeletal muscle and macrophages, high levels of CD36 have been correlated with insulin resistance (Bonen *et al.*, 2004; Liang *et al.*, 2004). However, this correlation may be due to the up-regulation of CD36 that occurs in response to high fat feeding (Greenwalt, Scheck and Rhinehart-Jones, 1995) and diabetes (Greenwalt, Scheck and Rhinehart-Jones, 1995; Van Nieuwenhoven, Van der Vusse and Glatz, 1996) as a consequence of the increase fatty acid utilisation, demonstrated in cell culture with long-chain fatty acid treatment (Sfeir *et al.*, 1997).

In a rat model of MetS using spontaneously hypertensive rats (SH-rats), in which expression levels of CD36 are undetectable in adipocyte plasma membranes, a diet rich in ω-3 PUFAs was shown to increase CD36 mRNA expression to comparable levels to those of Kyoto-Wistar rats (KW-rats) on a control diet (Alexander Aguilera *et al.*, 2006). Metabolic parameters of the SH-rats were increased compared to the KW-rats prior to diet changes; they had increased blood pressure, serum insulin, FFAs and triglyceride levels. After the diet intervention, the SH-rats fed ω-3 PUFAs had significantly lower serum insulin, FFAs, triglycerides, total cholesterol, HDL, LDL, and total lipids than the SH-rats on a canola-corn control diet, giving a metabolic profile similar to the KW-rats. Serum glucose measurements were not found to be different across the three groups. This study is an example of one gene which has altered expression levels following the addition of ω-3 PUFA to the diet.

Agreement with these results has been shown in cell culture, increased *CD36* mRNA expression as the result of separate EPA and DHA treatment has also been shown in THP-1 macrophages, in a dose-dependent manner (Vallvé *et al.*, 2002). However, when expression of CD36 was measured by assessing mRNA levels in PBMCs isolated from healthy human males and females who's diet had been supplemented with combined EPA (1.8g/day) and DHA (0.4g/day) for 26 weeks; a decrease in *CD36* mRNA expression was observed (Bouwens *et al.*, 2009). Using a micro-array, the expression of 900 genes uniquely changed as a result of the ω-3 PUFA supplementation compared to a placebo. A decrease in expression of genes involved in inflammatory pathways was observed. However, no changes in plasma C-Reactive protein (CPR), a measure of inflammation, were seen in this cohort and no other

measures of inflammation were investigated. The study also found a decrease in the expression of *PDK4*, *LTA4H*, *ADFP*, *CD14* and *HIF1a* (Bouwens *et al.*, 2009).

Another cell membrane bound protein important in the anti-inflammatory effects of ω-3 PUFAs is G-Protein-Coupled receptor 120 (GPR120). GPR120 functions as a receptor for unsaturated long chain free fatty acids. EPA and DHA have been found to exert their potent anti-inflammatory effects through GPR120 (Oh *et al.*, 2010). It was shown that GPR120 knockout mice did not show inhibition of inflammation from ω-3 supplementation, whereas the wild-type mice had reduced inflammation and enhanced insulin sensitivity as a result of the supplementation. Two non-synonymous variants of GPR120 have been found to be associated with diabetes; Ichimura et al. (2012) also found that in GPR120 knockout mice fed on a high fat diet showed increased body weight compared to the wild-type mice fed on the same diet.

Evidence suggests that one of the ways in which ω-3 PUFAs exert these effects is through changes in gene expression as a result of varied transcription factor activation (Bouwens *et al.*, 2009; Rudkowska *et al.*, 2013), however, they may also be a consequence of changes in epigenetic mechanisms, such as DNA methylation.

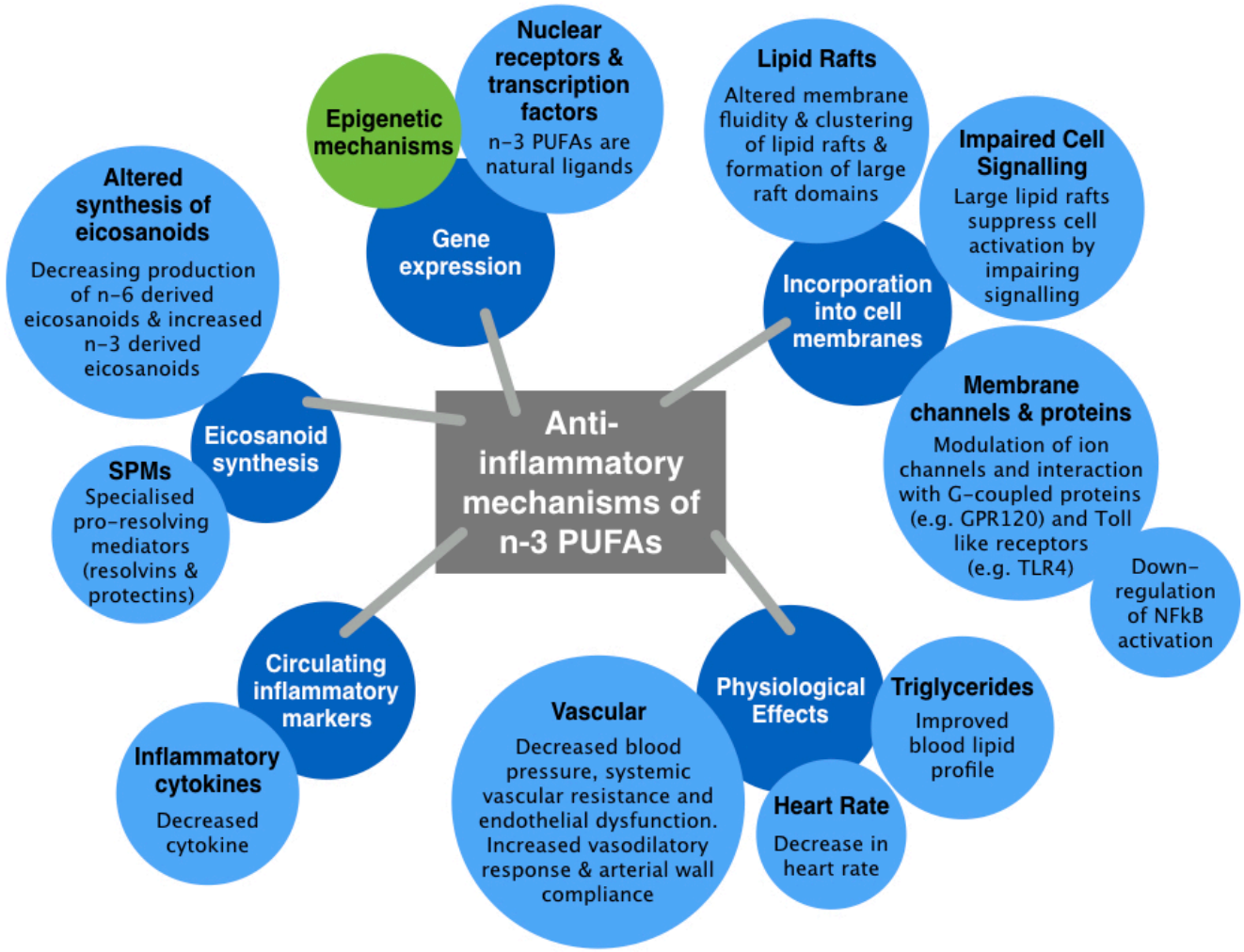

Figure 2-5 Mechanisms Omega 3 polyunsaturated fatty acids (ω-3 PUFAs) action to promote an anti-inflammatory environment. G-coupled protein receptor 120 (GPR120); nuclear factor kappa B (NFkB); omega 6 polyunsaturated fatty acids (ω-6 PUFAs); Specialised pro-resolving mediators (SPMs); toll like receptor 4 (TRL4).)

2.4 What is DNA Methylation?

DNA methylation is the most accessible and widely studied epigenetic mark (Lam *et al.*, 2012) and has been shown to vary with nutrition, disease and age (Choi and Friso, 2010; Gomes *et al.*, 2012; Bacalini *et al.*, 2014; Joyce *et al.*, 2015). DNA methylation occurs when a methyl group is covalently attached to the fifth position of the pyrimidine ring to give 5mC, Figure 2-6, and it is most commonly found on a cytosine (C) positioned next to a guanine (G; Jones and Takai, 2001), however methylation of adenine (A) is also observed in bacterial, plant and recently in mammalian DNA (Wu *et al.*, 2016; Xiao *et al.*, 2018). Within embryonic stem cells, a quarter of the methylation occurs at non-CG sites (Lister *et al.*, 2009), suggesting that there are different mechanisms for DNA methylation during development that do not endure into adulthood.

Methylation does not alter the underlying genetic code, and whilst it is a reversible process, it can also be passed through cell divisions (Kim *et al.*, 2010). DNA methylation is required for normal cell function, and it is also important because it can regulate gene expression, it is key to genomic imprinting, X-chromosome inactivation and repression of transposable elements (Bird, 2002).

In most cases, the result of DNA methylation is the silencing of genes by the inhibition of gene transcription, effectively deactivating the promoter region of the gene (Breiling and Lyko, 2015). It is thought that the methyl group inhibits transcriptional factor binding, interacts with methyl binding proteins and also alters the DNA shape by the methyl group protruding into the major groove of the DNA double helix and narrowing the minor grove (Lazarovici *et al.*, 2013). Therefore, the methylation level of DNA impacts upon the accessibility of the DNA, and in particular the gene promotors and regulatory regions. The addition of a methyl group results in transcriptional repression whilst the removal of a methyl group results in transcriptional activation.

Within the human genome, there are approximately 29 million CpG sites with 60-80% of these sites contain a methylation cytosine (Lister *et al.*, 2009). Regions of the genome that are enriched for CpG dinucleotides are termed CpG islands. These islands occur in various locations across the genome, 48% in promotors, 27% in

intergenic regions and 25% in gene bodies (Gao and Das, 2014). Most DNA methylation occurs in CpG sites that are dispersed across the genome, whereas CpG islands are predominantly unmethylated (Gao and Das, 2014).

The regulation of DNA methylation is completed by the DNA methyltransferase (DNMT) family of enzymes, where the methyl group is transferred from S-adenyl methionine (SAM) to the fifth carbon on the cytosine ring. Each enzyme plays a specific role: new methylation (*de novo*) transferred to the unmodified DNA is catalysed by DNMT3a, DNMT3b and cofactor DNMTL; DNA methylation patterns are maintained during DNA replication by DNMT1 (Prokhortchouk and Defossez, 2008). DNMT2 is not involved in DNA methylation but rather methylation of transfer ribonucleic acid (tRNA) (Goll *et al.*, 2006). The roles of the DNMT enzymes are reviewed in further detail by Gros et al. (2012) and Moore et al. (2013).

DNA methylation levels within the genome are cell- and tissue-specific, with changes observed during the human lifespan (Wilson *et al.*, 1987; Gilbert, 2009; Jones, Goodman and Kobor, 2015). Dysregulation of 5-methylcytosine (5mC) has been associated with diseases including cancer and cardiovascular disease (Ehrlich, 2002; Jones and Baylin, 2007; Muka *et al.*, 2016).

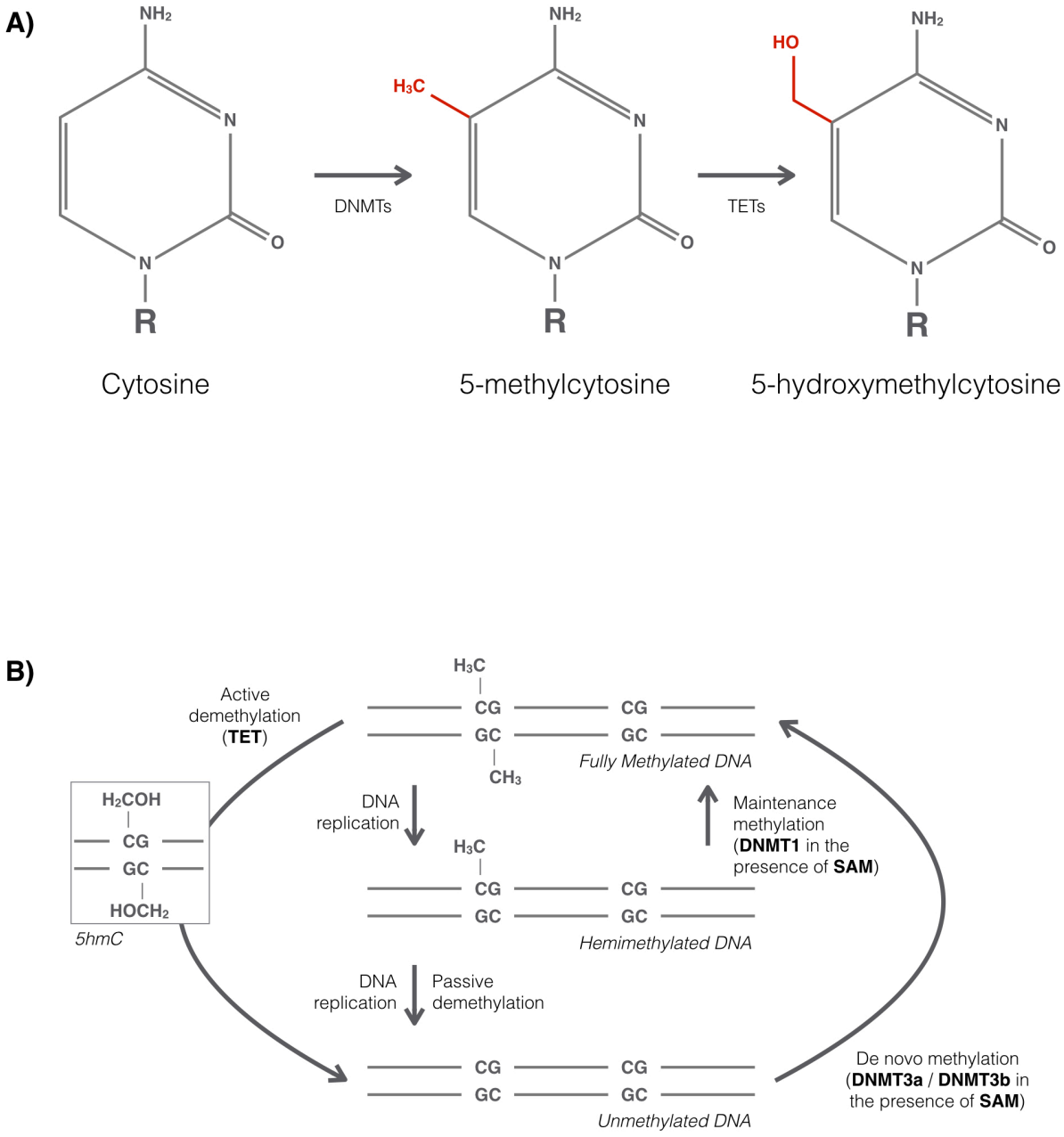

Figure 2-6 A) The chemical structure of cytosine and two of its modified forms, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). R group indicates connection to the DNA strand backbone; B) Cytosines positioned next to guanines (CG) can have a methyl group (CH3) covalently attached to the fifth carbon atom by DNA methyltransferase (DNMT) family of enzymes in the presence of co-factor S-adenosyl methionine (SAM) to give 5mC. DNA methylation in the form of 5-methylcytosine (5mC) can be actively reversed to unmodified cytosine (C) by the ten-eleven translocation (TET) family of enzymes, resulting cytosine modifications include 5hmC. Figure adapted from Chen and Riggs (2011)

2.5 Fatty Acids and Epigenetics

In this thesis the research is concerned with the interactions between fatty acids and DNA methylation. The following review of the literature aims to summarise the current knowledge of this relationship and identify where further research is required. The search strategy for the literature review involved combining the terms for DNA methylation (e.g. DNA methylation and CpG) with terms relating to fatty acids (e.g. saturated fatty acid, monounsaturated fatty acid polyunsaturated fatty acid, EPA and DHA). The online database PubMed was last searched using these terms on the 22nd May 2018. Articles were included in this review if they investigated the interactions between DNA methylation and fatty acids within humans and are summarised in Table 2-2.

2.5.1 Epigenome wide associations studies

Epigenome wide associations studies (EWAS), which despite the name, focus mainly on the specific epigenetic marker DNA methylation, have provided preliminary evidence linking ω-3 PUFA consumption with differential DNA methylation.

The *Yup'ik* people from Alaska provide a unique opportunity to study a population where there is a wide range in consumption of fish. Their traditional diet is rich in fish but this is changing with westernisation (Bersamin *et al.*, 2008), permitting stratification of this population into high and low ω-3 PUFA intake groups. One prominent EWAS of the *Yup'ik* (n = 185) utilised biochemical analysis to investigate associations between ω-3 PUFA status and DNA methylation; associations were observed at biologically relevant targets, including genes involved in T-cell homeostasis (Aslibekyan *et al.*, 2014). Positive relationships were observed in seventy eight percent of the associations with ω-3 PUFA status (Aslibekyan *et al.*, 2014). Genomic instability occurs with hypomethylation (Chen *et al.*, 1998), and therefore the increased methylation observed with high ω-3 PUFA status may be beneficial to the genomic stability.

In a study of a cohort of Greek preadolescents (n = 69), associations were found between dietary fats and DNA methylation, including sites within pathways linked to inflammation; nuclear factor kappa B (NFκB), peroxisome proliferator-activated

receptor alpha (PPARα), leptin and interleukin 6 (IL6*)* (Voisin *et al.*, 2015). This study measured dietary fat intake using FFQs. Although their results were consistent with the finding of Aslibekyan *et al.* (2014), in their study of the *Yup'ik* people, it is worth noting that in general, whilst FFQs provide an estimation of fatty acid intake, they do not provide a direct measurement of biologically available ω-3 PUFAs in the target tissue. It is the fatty acids within the target tissue that contributes to the environment of the cell, thus influencing the epigenetic control of the genome, rather than the diet itself.

The study of the *Yup'ik* cohort also found that individuals with higher ω-3 PUFA status had significantly lower levels of plasma triglyceride (TG), increased high density lipoproteins (HDL), and higher but not significant levels of low density lipoproteins (LDL) and total cholesterol (Aslibekyan *et al.*, 2014). Consistent with previous supplementation studies (Harris, 1997; Das, 2000) and dietary salmon consumption (Raatz *et al.*, 2016). It has been demonstrated that there is an association between blood lipids (TG, HDL and LDL) and DNA methylation of the genes encoding six key regulators of lipid metabolism (Dekkers *et al.*, 2016), and therefore the DNA methylation associations observed in the studies discussed above may not be due to ω-3 PUFAs in the diet, but rather differences in blood lipid profiles, or a combination of both. One method of addressing this issue is the utilisation of closely controlled intervention studies.

As briefly discussed in Chapter 1, the impact of nutrition during early life is of interest due to the plasticity of the epigenome during development and the links to diseases in later life (Gluckman and Hanson, 2004b; Gluckman *et al.*, 2008).

Global methylation analysis pre- and post-intervention can be used to investigate the impact of ω-3 PUFA supplementation on differentially methylated regions (DMRs) throughout the genome. The first two intervention studies that are considerd took slightly different approaches, the first supplemented mothers with 3.7g/day EPA+DHA (n = 36) or placebo (n = 34, placebo details not provided) from 20 weeks of gestation to delivery (Amarasekera *et al.*, 2014). Whereas, the second study supplemented 9-month-old infants with either 1.6g/day EPA+DHA (n = 6) or a placebo 3.1g/day LA (n = 6, LA in the form of sunflower oil); (Lind *et al.*, 2015). Neither investigation found a

significant difference in DNA methylation between those taking the ω-3 PUFA and those taking the placebo after adjusting for multiple testing (Amarasekera *et al.*, 2014; Lind *et al.*, 2015). In contrast, however, a third more recent large randomised control trial (RCT) supplementing mothers during gestation with 1.5g/day ω-3 PUFA (0.8g/day DHA + 0.1g/day EPA, n = 190) or 1.5g/day vegetable oil placebo (n = 179), found that there were significant DMRs, including those relating to immune function, between the two experimental groups (van Dijk *et al.*, 2016).

The apparent differences in results between the first two studies and the third warrant consideration. Cohort size in the first two studies was small as compared to the larger third study. It is possible that the differences in methylation are so small that significant results only become apparent in a larger sample size. In addition, a confounding factor might also be that the relationship between methylation and supplementation are sex specific and could, at least in part, cancel each other out. In fact it is known that there are significant differences in the way males and females metabolise and store ω-3 PUFAs (Burdge and Wootton, 2002; Burdge, Jones and Wootton, 2002; Lohner *et al.*, 2013). It has also been shown that there are differences in global methylation levels between the sexes (Zhang, Cardarelli, *et al.*, 2011); which is consistent with the clear effect observed between sexes in the third study where there was higher number of differentially methylated regions (DMRs) in males compared to females (van Dijk *et al.*, 2016). It is therefore important to appreciate that when using small sample sizes for ω-3 PUFA and DNA methylation studies, a lack of separation of the sexes may result in critical results being overlooked.

Notably, the majority of studies presented thus far have utilised Illumina Infinium HumanMethylation450 BeadChip Arrays™ (450k arrays). This technology measures DNA methylation at over 450,000 individual CpG sites throughout the genome, and although a powerful tool in explorative work in determining DMRs, there are limitations to the use of 450k array data, including multiple testing, the generation of artefactual data and the need for complicated normalisation (Dedeurwaerder *et al.*, 2014). Therefore, targeted analysis of specific CpG sites through a candidate gene approach should be completed to confirm the 450k array findings and provide additional information about methylation at specific and relevant sections of DNA.

2.5.2 LINE1 as an estimation of Global Methylation

It has been estimated that repetitive transposable elements account for 45% of the genome (Lander *et al.*, 2001) and it is estimated that these transposable elements contain one third of the genome's DNA methylation (Kochanek, Renz and Doerfler, 1993; Schmid, 1998). Measuring the levels of DNA methylation in repetitive elements, such as long interspersed nucleotide elements (LINEs), can therefore provide a proxy for global DNA methylation (Zhu *et al.*, 2012).

The high level of methylation in these elements represses their transcription (Kemp and Longworth, 2015). Associations between *LINE1* methylation and lifestyle factors, including physical activity and nutrition have been found (Zhang, Cardarelli, *et al.*, 2011; Zhang, Morabia, *et al.*, 2011; Agodi *et al.*, 2015; Garcia-Lacarte *et al.*, 2016; Marques-Rocha *et al.*, 2016) and have also been shown to be associated with both disease (Schulz, Steinhoff and Florl, 2006; Baccarelli *et al.*, 2010; Joyce *et al.*, 2016) and ageing (Bollati *et al.*, 2009; Jintaridth and Mutirangura, 2010).

DNA methylation of *LINE1* in cord blood mononuclear cells (CBMCs) was measured from mothers who received 0.4g/day DHA (n = 131) or placebo (n = 130, olive oil) during gestation (Lee *et al.*, 2013). Neither this study or the third RCT above found any differences in *LINE1* methylation between supplementation and control groups (Lee *et al.*, 2013; van Dijk *et al.*, 2016). However, Lee *et al.* (2013) did find an interaction between smoking status and DHA supplementation; CBMCs of mothers who smoked and took DHA supplements during gestation had significantly higher *LINE1* methylation levels than the CBMCs of mothers who smoked and took the control supplement. These results suggest that there is a complex interaction between multiple environmental stimuli and the epigenome.

2.5.3 Candidate Targets

Whilst LINE1 analysis provides information about DNA methylation status across the genome, it does not provide the level of detail that is required to fully understand what is happening at a gene level. In order to investigate the gene environment interaction in more detail it is necessary to study methylation of specific regions within genes that are thought to be relevant to transcriptional activity.

In addition to *LINE1* methylation, Lee *et al.* (2013) investigated specific genes and found no significant differences in methylation of *TNF* "tumor necrosis factor", *IL13 "*interleukin 13", *GATA3* "GATA binding protein 3", *STAT3 "*signal transducer and activator of transcription", *IL10* "interleukin 10" and *FOXP3* "forkhead box P3". However, the promotor methylation was lower, although not significantly, for *IFNG* "interferon gamma" in CBMC from participants supplemented with DHA (Lee *et al.*, 2013). This study was novel in that the supplementation only contained DHA. It would have been interesting to see if they had also included a separate EPA supplement group in order to establish whether there are differing effects on the epigenome. Unfortunately the comparison between EPA and DHA is often lacking in *in vivo* human studies potentially because of the increased cost of multiple intervention groups and a greater availability of mixed ω-3 PUFA supplements commercially. Despite this, *in vitro* studies do provide evidence of differing effects of EPA and DHA on inflammation (Gorjão *et al.*, 2009; Mickleborough *et al.*, 2009).

In terms of genes relevant to the inflammatory process, two have been investigated briefly in cross-sectional association studies. The methylation status of an individual CpG site within *IL6* was found to be negatively associated with ω-3 DHA and total ω-3 fatty acids (Ma *et al.*, 2016). The relationship was dependent on the genotype of a nearby single nucleotide polymorphisms (SNPs; Ma *et al.*, 2016). Anthropometric measurements, blood lipids and levels of ω-6 fatty acids, all contribute to explaining the variance in methylation status of *TNF*, an inflammatory cytokine encoding gene (Hermsdorff *et al.*, 2013); similar associations have been observed in the clock circadian regulator genes: CLOCK, BMAL1 and PER2 (Milagro *et al.*, 2012).

We have already noted that fatty acids are important structural components of cell membranes (Section 2.2.1). Consumption of foods rich in ω-3 PUFAs, such as salmon, results in increased incorporation and modification of cell phospholipid membrane (Raatz *et al.*, 2016). Composition of the membrane is altered by clustering of lipids rafts containing the ω-3 PUFAs, resulting in the formation of large raft domains (Turk and Chapkin, 2013). As shown in murine obesity models (Kim *et al.*, 2008) and HeLa cells (Chapkin *et al.*, 2008) large lipid rafts can suppress the cell activation by impaired signalling, ultimately affecting cell function through suppression of downstream pathways, including those involved in inflammation.

Leptin, a protein involved in energy balance, is localised to these lipid rafts and its expression is reduced by ω-3 PUFAs (Reseland *et al.*, 2001; Klok, Jakobsdottir and Drent, 2007). This reduction in expression may be a result of epigenetic control of the gene. Indeed epigenetic control of the *LEP* "leptin" gene has been demonstrated in a murine model (Shen *et al.*, 2014). However, when using a murine model candidate gene approach to investigate the effect of ω-3 PUFA supplementation on epigenetic control, no effect on *LEP*, *LEPR* "leptin receptor" or *POMC* "pro-opiomelanocortin" promotor methylation was found (Fan *et al.*, 2011). In humans, cross-sectional evidence indicates differential methylation in the leptin pathway genes with varying ω-3 PUFA status (Voisin *et al.*, 2015). Whether or not supplementation of the human diet would alter methylation status of these genes remains to be seen, but if it does then the results would be contrary to those found in the murine model.

Another protein found in cell membranes, *CD36* , also has its expression altered by fatty acid treatment *in vitro* (Vallvé *et al.*, 2002) and in animal models (Alexander Aguilera *et al.*, 2006). This membrane glycoprotein promotes inflammation in monocytes and macrophages. In addition it binds long chain fatty acids to facilitate their transport into the cell (Silverstein and Febbraio, 2009). In humans, the methylation of *CD36* promotor was significantly reduced by a the low-calorie diet, and this reduction was attenuated by ω-3 PUFA supplementation (Amaral *et al.*, 2014).

However, decreases in DNA methylation of other genes investigated within the same study, *CD14* "cluster of differentiation 14" and *PDK4* "pyruvate dehydrogenase kinase 4" were shown to be only significant as a result of the low-calorie diet and were not

attenuated by the ω-3 PUFA supplementation. Gene expression was not measured in this study. When the effect of ω-3 PUFA supplementation has been investigated on the expression of these genes in peripheral blood mononuclear cells (PBMCs), a decreased expression of *CD36*, *CD14* and *PDK4* was observed (Bouwens *et al.*, 2009). It is possible that the low calorie diet impacted on the DNA methylation to a greater extent than the ω-3 PUFAs, which only attenuated the decrease in methylation of *CD36* once adjusted for baseline body weight. This study illustrates the difficulty in understanding the relationships between ω-3 PUFAs and methylation when there are confounding factors, such as multiple interventions.

In fact the study carried out by Amaral *et al.* (2014) included an additional confounding factor because the participants were asked not to consume seafood and to follow a detailed diet plan during the intervention period to minimise variability. However, this study was carried out in Spanish females. The Spanish diet is high in shellfish/fish, on average 88.6g/person/day are consumed (Varela-Moreiras *et al.*, 2013), and therefore the researchers may have simply replaced the ω-3 PUFAs that the participants obtained from their habitual diets with the supplementation. No biochemical measurements for EPA and DHA were provided in this paper (Amaral *et al.*, 2014), however, earlier work from the same researchers for this cohort described the EPA and DHA increased after the intervention (Parra *et al.*, 2008). In order to be certain about the effects of supplementation it is important to include measurement of ω-3 PUFA in the tissue used for epigenetic analysis both before and after the intervention in order to confirm changes to the ω-3 PUFA status of the cells investigated.

Endogenous metabolism of PUFAs occurs within a cell. The initial step in the metabolic pathway takes places in the endoplasmic reticulum, where LA and ALA undergo elongation by fatty acid enlongase (ELOVL) and desaturation by Δ6-desaturase and Δ5-desaturase (Benatti *et al.*, 2004). The Δ6-desaturase and Δ5-desaturase enzymes, encoded by the *FADS2* and *FADS1* genes respectively, are rate limiting steps in this metabolic pathway (Marquardt *et al.*, 2000; Nakamura and Nara, 2004). Genetic polymorphisms of these genes and others related to PUFA metabolism can significantly alter the levels of PUFAs within the body (Glaser, Heinrich and Koletzko, 2010) and differential methylation is likely to have a similar effect. Associations between methylation of genes encoding fatty acid metabolism enzymes

and fatty acids levels have been found in multiple cohorts; these relationships were found to be altered by the genotype of relevant SNPs (Cui *et al.*, 2016; Rahbar *et al.*, 2018). Furthermore, the methylation of *FADS1* is altered by a low calorie diet with a small interaction with ω-3 PUFA supplementation (Amaral *et al.*, 2014). When investigated alone, with a higher dose of ω-3 PUFA supplementation, no significant changes in methylation were observed in *FADS1* or the elongation gene *ELOVL2* (Hoile *et al.*, 2014). However, the methylation of two other fatty acid metabolism genes, *FADS2* and *ELOVL5*, increased with supplementation; with a larger number of CpG sites changing methylation in females than males and a negative relationship between the DNA methylation and the gene messenger RNA (mRNA) levels was observed (Hoile *et al.*, 2014).

Table 2-2 Summary of literature investigating fatty acids, with a particular focus on DNA methylation, in human participants. See abbreviation list on page XV.

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
Amarasekera *et al.*, (2014)	Mother-infant pairs (n=70, 31-32 yrs) who were atopic and had clinical allergy [Male (n=37) and female (n=33) Infants]	DB RCT ~18 weeks supplementation (20 weeks' gestation to birth) with Fish oil (n=36; 3700mg/day) or control (n=34; olive oil);	RBC fatty acid profile (maternal and cord blood)	CD4+ T-cells from cord blood Infinium 450 array	No significant associations found with ω-3 PUFA levels after multiple testing. A dose-response was observed in the top ranked unadjusted CpG sites and the total ω-3 fatty acid levels.
Arpón *et al.*, (2017)	Male (n=18, 55-60 yrs) and female (n=18, 60-80 yrs) healthy adults	RCT 5 years Mediterranean diet with olive oil (n=12) or with nuts (n=12) or control diet (n=12; advice to reduce fats)	No measurement	Buffy Coat Infinium 450 array	Specific CpG sites showed differential methylation after five years of MedDiet with additional fats from either nuts or olive oil compared to control diet. Pathways analysis showed differential methylation in genes relating to metabolism, inflammation, intracellular signals and diabetes phenomena. Methylation of a CpG site within CPT1B gene was associated with PUFA uptake

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
Aslibekyan *et al.*, (2014)	Male (n=91) and Females (n=94) young adults of Yup'ik descent	Cross-sectional, extreme phenotype High and Low ω-3 PUFA status	RBC ω-3 PUFA status (nitrogen stable isotope ratio (d15N))	Whole blood Infinium 450 array	A total of 27 disease related sites significantly correlated with ω-3 PUFA (not after adjusting for multiple testing); 78% of significant associations were increased methylation. Inflammatory related methylated CpG sites associated with d15N included *CCL17*, *FAS*, *EIF2AK4*. A significant difference in age between the two groups.
Cui *et al.*, (2016)	Male Prostate Cancer patients (n=60, 55-64 yrs)	Cross-sectional	Prostate tumour fatty acid profile	Prostate tumour *FADS* cluster (cg27386236)	Methylation of cg27386326 negatively associated with Arachidonic acid, along with ratio ARA+ADA to LA (indicating ω-6 biosynthesis). Genotype of rs174537 significantly interacts with fatty acids (stearic, oleic, linoleic, DGLA, ARA, Adrenic, DPA, DHA and ω-6:ω-3 ratio).
do Amaral *et al.*, (2014)	Overweight females (n=12, 20-40 yrs)	DB RCT 8 weeks calorie restriction (30%) with supplementation of ω-3 PUFAs (n=7; >1300mg/day) or Control (n=5; ω-3 PUFAs <260mg/day)	Blood lipid profile (total cholesterol, LDL, HDL, TAG)	PBMC *CD36*, *CD14*, *FADS1*, *PDK4* and *FFAR3*	Weight loss similar between groups. Diet reduced DNA methylation of CD36 (CpG+477, -11.8% control & -7.3% ω-3 PUFA), no influence of fish oil unless baseline body weight was controlled for in which case methylation was lower in ω-3 PUFA group. Small increase in CD14 methylation (CpG +765+733, +0.3% control & +0.6% ω-3 PUFA). Significant, but small changes in promotor methylation *PDK4* (CpG -222-50) and *FADS1* (CpG -25-22-20); methylation percentage of *PDK4* and *FADS1* reduced in control group and increased in fish oil group. No data on gene expression. Leptin significantly changed with weight loss, no interaction and no fish oil significance. No significant change in blood lipid profiles.

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
Hermsdorff *et al.*, (2013)	Female healthy young adults (n=40, 18-28 yrs)	Cross-sectional, extreme phenotype High and Low truncal fat	Blood lipid profile (total cholesterol, LDL, HDL, TAG); Semi-quantitative food frequency questionnaire (136 food-items)	Buffy coat *TNF*	Higher inflammation (TNFα and CRP) in high truncal fat women. Methylation status of 2 CpG sites within the exon 1 *TNF* gene significantly different between low and high truncal fat. Methylation of one and average of both sites negatively correlates with plasma TNFα; with truncal fat positively correlates with plasma TNFα. Central adiposity indicator (truncal fat), metabolic features (HDL-c and insulin), plasma TNFα, and dietary fat intake (ω-6 fatty acid) all contribute to explaining 48% of the variation in methylation state of one CpG site in *TNF*.
Hoile *et al.*, (2014) **Cohort 1** **Australia**	Male (n=16) and Female (n=13) Chronic kidney disease patients (47-64 yrs)	DB RCT 8 weeks supplementation with ω-3 PUFA (n=14, EPA 1800mg, DPA 200mg, DHA 1500mg/day) or Olive oil (n=15, 4000mg/day)	No measurement	PBMC *FADS1*, *FADS2*, *ELOVL2*, *ELOVL5*	Both ω-9 PUFA and ω-6 PUFA supplementation induced changes to methylation status of four *FADS2* CpG site and one *ELOV5* CPG site that were both sex specific and specific to the PUFA supplemented. These sites were negatively associated with *FADS2* and *ELOV5* mRNA expression.
Hoile *et al.*, (2014) **Cohort 2** **England**	Male (n=8) and Female (n=12) healthy adults (22-34 yrs)	Supplement 12 weeks supplementation of ω-3 PUFA (n=20, EPA 1100mg, DPA 100mg, DHA 800mg/day)	No measurement	Whole blood *FADS1*, *FADS2*, *ELOVL2*, *ELOVL5*	Results for all bar two of the CpG sites were replicated from cohort 1 ω-3 PUFA group.

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
Karimi *et al.*, (2017)	Male (n=32) and Female (n=31) Mild to moderate Alzheimer's patients (73±9 yrs)	DB RCT 6 months supplementation of ω-3 PUFA (n=30, EPA 600mg, DHA 1700mg/day) or Placebo (n=33, Corn oil 1000mg, linoleic acid 600mg/day)	Plasma fatty acid profile	Buffy coat *LINE1*	Significant decrease in *LINE1* methylation with ω-3 PUFA supplementation, observed in both males and females. Significant differences to the placebo group observed after six months supplementation, the placebo increasing methylation of *LINE1*.
Lee *et al.*, (2013)	Mother-infant pairs (n=261, 18-35 yrs) who were atopic and had clinical allergy. [Male (n=133) and female (n=128) Infants]	DB RCT 14-18 weeks supplementation (18-22 weeks' gestation to birth) with 400mg/day DHA (n=131) or control (olive oil, n=130)	No measurement	CBMC *IFNG, TNF, IL13, GATA3, STAT3, IL10, FOXP3* & *LINE1*	No difference in *LINE1* methylation between the ω-3 PUFA and control groups, the interaction between smoking status of mothers and ω-3 PUFAs was significant, with higher methylation in the ω-3 PUFA smoking mothers group (n=26). *IFNG* promotor methylation was lower (not significantly) in the ω-3 PUFA group. No effect was observed in *TNF, IL13, GATA3, STAT3, IL10* and *FOXP3*. ω-3 PUFA supplementation altered the ratio of methylation levels between *IFNG* and *IL13* (Th1-Th2 specific genes).

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
Lee *et al.*, (2014)	Mother-infant pairs (n=261, 18-35 yrs) who were atopic and had clinical allergy. [Male (n=133) and female (n=128) Infants]	DB RCT 14-18 weeks supplementation (18-22 weeks' gestation to birth) with 400mg/day DHA (n=131) or control (corn & soy oil, n=130)	No measurement	CBMC *IGF2* & *H19*	Modulation of methylation status of *IGF2* and *H19* DMRs by DHA supplementation, dependent on pre-pregnancy maternal BMI. Interactions between maternal BMI and *IGF2* DMR, and *H19* DMR methylation. Increased methylation of *IGF2* in preterm infants who received DHA supplementation. (Note: discrepancy in description of control oil for same cohort as Lee *et al.*, (2013) above)
Lind *et al.*, (2015)	Male (n=6) and Female (n=6) full term infants (9 mths)	DB CT 9 months supplementation of ω-3 PUFAs (n=6, 1600mg EPA+DHA) or sunflower oil (n=6, 3100mg)	RBC fatty acid profile	Buffy coat Infinium 450 array	ω-3 PUFA group had lower ex vivo production of IL6 than the control group (p=0.08). No discrimination in DNA methylation between the two interventions after adjusting for multiple testing. 43 CpG sites had a >10% difference in absolute methylation, including sites in transcription factors (*GATA4, TBPL2*), nucleic acid binding proteins (*RBM9*, *CDYL*) and metabolic genes (*ACOT13, KCNV1, NR3C1*).
Ma *et al.*, (2016)	Male (n=459) and Female (n=389) adults	Cross-sectional	RBC fatty acid profile	CD4+ T-cells *IL6* (cg01770232, cg26061582)	Higher methylation of cg01770232 associated with higher IL6 gene expression and higher plasma IL6. Negative association between total ω-3 PUFAs and cg01770232 methylation (dependent on rs2961298 genotype), as well as plasma IL6.

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
McMorrow *et al.*, (2018)	Male and Female overweight and obese adolescents (n=70, 13-18 yrs)	DB RCT(crossover) 8 weeks supplementation of AINS [LC ω-3 PUFA (1000mg EPA and 1000mg DHA), 567mg vitamin C, 390mg α-tocopherol, 416mg green tea extract (45% epigallocatechin ω-3-gallate) and 16.5mg lycopene.] or Placebo (oleic sunflower oil and microcrystalline cellulose), 6 weeks washout, 8 weeks opposite supplement	Blood lipid profile (total cholesterol, LDL, HDL, TAG)	Buffy coat Infinium 450 array	Significant association between adiponectin and methylation of 487 CpG sites following AINS supplementation. Pathway analysis identified positive associations with pathways including metabolic gene, genes involved in developmental biology and gene express, as well as an inverse relationship with genes involved in signal transduction and the immune system.
Milagro *et al.*, (2012)	Female adults (n=60, 23-53 yrs) [normal weight (n=20), overweight/obese (n=20) and morbidly obese (n=20)]	Methylation only measured at baseline 16-week Weight reduction intervention	Blood lipid profile (total cholesterol, LDL, HDL, TAG)	PBMC *CLOCK-R1, CLOCK-R2, BMAL1* and *PER2* (measured at baseline only)	The methylation of *CLOCK* showed associations with the intake of MUFA and PUFAs. Associations with methylation of CpG sites located in clock genes (*CLOCK, BMAL1* and *PER2*) with obesity, MetS and weight loss.

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
Perfilyev et al. (2017)	Male (n=22) and Female (n=9) healthy adults (20-38yrs)	DB RCT 7 weeks of overfeeding (high calorie muffins) containing either:	Blood lipid profile (total cholesterol, LDL, HDL, TAG)	Adipose tissue Illumina 450K array	Mean methylation of 1797 genes (including *FTO, IL6, INSR, NEGR1, POMC*) was changed by PUFA overfeeding compared to 125 genes by SFA overfeeding. When data combined for PUFA and SFA, mean methylation of 1444 genes was changed by overfeeding, including *FABP1*, *FABP2, MC2R, MC3R, PPARGC1A* and *TNF*. The PUFA diet did not significantly alter gene expression, compared to the SFA which altered the expression of 28 transcripts.
Rahbar *et al.*, (2018) **Cohort 1**	Male (n=31) and Female (n=58) healthy adults (21-65 yrs)	Cross-sectional	Serum fatty acid profile	Leukocytes *FADS1*, *FADS2*	*FADS2* methylation an indicator of circulating ω-6 PUFA. DNA methylation in the *FADS2* promotor were inversely related to DGLA levels. Positive association between *FADS2* methylation and AA/DGLA ratio. Allele specific methylation with rs174537 and *FADS2* methylation.
Rahbar *et al.*, (2018) **Cohort 2**	Male (n=13) and Female (n=19) healthy adults (21-65 yrs)	Cross-sectional	Plasma fatty acid profile	CD4+ Cells and Saliva *FADS1*, *FADS2*	Consistent findings as cohort 1 for CD4+ cells methylation of *FADS2* and inverse relationship with DGLA levels. Positive association between *FADS2* methylation and AA/DGLA ratio. No associations observed in saliva methylation and circulating fatty acids.
Tremblay *et al.*, (2017)	Male (n=18) and Female (n=18) overweight and obese adults (34.7 ± 8.8 yrs)	Supplement 6 weeks supplementation with 3000mg ω-3 PUFA (1900-2200mg EPA, 1100mg DHA)	Plasma fatty acid profile; Blood lipid profile (total cholesterol, VLDL, LDL, HDL, TAG)	Whole blood Illumina 450K array	Post supplementation, 308 CpG sites, relating to 231 genes, were differentially methylated, 93% (286 CpG sites) were hypermethylated post supplementation. Total of 55 overrepresented pathways altered post supplementation, including 16 that were related to inflammatory and immune response, lipid metabolism, type 2 diabetes, and cardiovascular signalling.

Reference	Cohort	Study type / Intervention	Fatty acid	Tissue / Targets	Results
van Dijk *et al.*, (2016)	Mother-infant pairs (n= 991, ~30 yrs) who were healthy [Male (n=502) and female (n=489) Infants]	DB RCT 18+ week supplementation (<21 weeks' gestation to birth) with 1500mg ω- PUFA (800mg DHA and 100mg EPA) or 1500mg vegetable oil (rapeseed, sunflower, and palm)	Plasma DHA	Neonates: Dried blood spots Age 5 yrs: Whole blood *LINE1*, (n=991 neonates, n=663 age 5 yrs) Infinium 450 array (n=369 neonates, n=65 age 5 yrs)	*LINE1* methylation higher in males than females. No significant difference in *LINE1* methylation between intervention groups at birth or 5 years. After DHA supplementation compared to the control group there were 21 differentially methylated regions, predominantly lower methylation, genes included ones involved in lipid exchange between membranes (*ESYT3*), plasma membrane function (*SLC12A6*), appetite regulation (*CCK*), immune function (*RAET1L* and *LTB*) and neurodevelopment/brain function (*SLC12A6, TRAK1, LPHN3* and *RFPL2*). Limited overlap with significant DMRs at birth and 5 years.
Voisin *et al.*, (2015)	Male (n=22) and Female (n=47) Greek preadolescents [Normal-weight (n=34), Obese (n=35)]	Cross-sectional	Dietary intake via 24hr recall for 2 weekdays and 1 weekend day	Whole blood Infinium 450 array	34 pathways significant for PUFA/SFA diet content including adipogenesis, *PPARγ*, leptin and IL6 pathways. Five pathways significant for (MUFA+PUFA)/SFA diet content including ones linked to *NFkB*.

The literature review performed here highlights the varied nature of the studies investigating ω-3 PUFAs and DNA methylation performed to date. The lack of consistency of the study designs makes it difficult to interpret the relationship and indicate points to consider when conducting the research being undertaken in this thesis.

Firstly, there is not yet a consensus on the dosage level of ω-3 PUFAs required to produce an anti-inflammatory effect in humans. In a review by Calder (2015), it is suggested that a dose between 1.35g/day and 2.7g/day of EPA is required. In addition, there is a dose response relationship between ω-3 PUFAs and the effect on cytokine levels (Caughey *et al.*, 1996), it is possible that there will also be a dose-response relationship in epigenetic control of inflammation.

There is also no consensus on the timeframe required for epigenetic changes to occur. The largest incorporation of ω-3 PUFAs into erythrocyte membranes occurs within the first 4 weeks of supplementation and it is not known if the epigenetic changes occur within the same timeframe.

Most studies measure fatty acid content of RBCs or plasma, with fewer measuring the incorporation into PBMCs which are used as the source of DNA in many of the methylation studies. In addition, none of the studies consider methylation alongside fatty acid incorporation into PBMCs.

Finally, the majority of intervention studies investigating epigenetic changes do not account for the impact of the intervention on the cell population from which the DNA was extracted. This results in multiple complications; firstly, if the influence of ω-3 PUFAs is specific to one cell type it may not be observed if the cells are not separated prior to analysis. Secondly, changes may be falsely observed or masked with variations in cell populations. Differentiation into the cell subtypes found within blood requires changes within the epigenetic signatures, and therefore the observed changes in percentage methylation for an individual CpG may be the result of a change in the cell population rather than a result of the intervention. Data can be normalised to account for the proportion of different cells (Hoile *et al.*, 2014; Lind *et al.*, 2015).

2.6 Conclusion

Associations between fatty acids and DNA methylation have been demonstrated, with further evidence that supplementation with fatty acids, such as ω-3 PUFAs, affect methylation levels of certain genes some of which are involved in inflammation. A more targeted approach to the effects of ω-3 PUFAs, and use of well controlled supplementation studies could elucidate a mechanism through which ω-3 PUFAs alter the DNA methylation within the inflammatory landscape.

Having regard to the importance of ω-3 and ω-6 PUFAs in health and disease and the recent studies that have observed differences in DNA methylation dependent on PUFA levels, the general aims of this research are to:

- Characterise the relationship between methylation of a candidate cytokine gene and the fatty acid composition in blood;
- Identify if changing the fatty acid composition of PBMCs can alter the methylation signatures of selected cytokine genes;
- Investigate whether the supplementation of ω-3 PUFAs reduces the inflammation caused by eccentric exercise induced muscle damage through alteration of the methylation status of the cytokine genes.

The null hypotheses tested within this thesis:

- The is no relationship between methylation of a candidate cytokine gene and the fatty acid composition in blood;
- Changing the fatty acid composition of PBMCs does not alter the methylation signatures of selected cytokine genes;
- Supplementation of ω-3 PUFAs does not reduce the inflammation caused by eccentric exercise induced muscle damage through alteration of the methylation status of the cytokine genes.

Chapter 3 Method Development: Quantification of DNA Methylation

3.1 Introduction

The genetic analysis of DNA sequences has come a long way since 1977 when Frederick Sanger first published details of gel-based enzymatic termination sequencing (Sanger, Nicklen and Coulson, 1977), which today is known as Sanger Sequencing and was the work horse of the Human Genome Project referred to in the introduction of this thesis. Since the advent of Sanger sequencing and the subsequent developments in sequencing and molecular biology technologies (Heather and Chain, 2016), give scientists the ability to analyse the epigenetic code of cells more easily, at less cost and in greater depth than ever before.

This chapter explains how DNA methylation will be measured in this research and provides details of the design and validation of assays used later in the thesis.

3.2 Measuring DNA methylation

3.2.1 Method selection

DNA methylation was first discovered in 1948 (Hotchkiss, 1948), since then there has been an evolution in the methods that can be used to measure this epigenetic mark. Measurement of cytosine methylation levels involves either the use of methylation-specific restriction enzymes, bisulphite conversion of DNA, or enrichment with methylation targeting enzymes. Methodologies fall into two categories, epigenome - wide or locus-specific.

Levels of methylation across the genome can be measured in a non-locus specific manner by global methylation analysis techniques which include luminometric methylation assay (LUMA), enzyme-linked immunosorbent assays (ELISAs), high performance liquid chromatography (HPLC), repetitive element DNA methylation analysis (e.g. LINE1) (Kurdyukov and Bullock, 2016). However, these techniques do not provide information on the levels of methylation at certain loci. This may lead to losing key information on the methylation patterns in certain loci of interest. Measurement techniques which allow for the identification of methylation at individual loci include bisulphite whole genome sequencing (BS-WGS), which provides details of methylation levels across the whole genome; or regional levels can be measured via pyrosequencing. Epigenome-wide methylation analysis determines global methylation levels, examples of such methods include high performance liquid chromatography (HPLC) and bisulphite whole genome sequencing (BS-WGS); the latter of which also provides detail on the location of the methylation marks. There are methods, such as array based chips, which give levels of methylation from loci spread across the genome, representing approximately 2% of the sites that could be methyalted.

Methods which do not allow for measurement of methylation at individual loci can be difficult to link changes to biologically significant outcomes, however, the cost of the global methylation methods of £5 per sample is significantly cheaper than the genome wide loci specific methods. The use of Illumina Infinium HumanMethylation450 BeadChip Arrays™ (approx. £250/sample) and BS-WGS (approx. £3,600/sample) do,

however, provide information on individual CpG sites. These methods are often used to scan the genome for novel DMRs. However, in addition to their high cost, they require large amounts of input DNA and even then, significant results found using the 450k array should be validated using sequencing-based methods. Microarrays, such as the 450k array, are susceptible to batch effects and require comprehensive analytical techniques to correct for this. In addition, due to the requirement to have two different probes types on the same array, to be able to still measure methylation in areas of high CpG density, data processing can be problematic as a result of the differences in the performance of the probes (Pidsley *et al.*, 2013).

An alternative to epigenome-wide methylation analysis, which can be used when a candidate gene has been identified, is the measurement of methylation levels of smaller regions of DNA. The methylation of these smaller regions can be quantified after bisulphite conversion using polymerase chain reaction (PCR)-based methods, which include methylation sensitive PCR (MSP), MethyLight (utilising real-time PCR), methylation-specific high-resolution melting and bisulphite pyrosequencing.

Methylation sensitive PCR permits screening of methylation at a specific site; however, it is not quantitative. Real-time PCR methods do allow quantitative measurement at specific sites and utilise primer sets designed to amplify methylated or unmethylated regions; however, this will only allow for the detection of completely methylated or unmethylated sites within the target region.

Having regard to the advantages and disadvantages of the various available methods, including budgetary constraints, and having considered the number of samples that would have to be analysed during the course of the proposed research the decision was made to use a PCR-based method for analysing DNA methylation at specific identified CpG sites of interest.

Methylation levels of CpG sites within a specific region can be assessed by using the primer extension sequencing method, pyrosequencing. Pyrosequencing uses bisulphite-converted amplified DNA and provides sequence data over short DNA stretches. Methylation can be quantified by calculating the ratio of unconverted cytosine to converted thymine at the CpG loci (see below for further details on bisulphite conversion). Benefits of the pyrosequencing method include the inclusion of

an internal bisulphite conversion control and the ability to measure individual methylation values of CpG sites in the short stretch of DNA sequenced. As the length of DNA sequenced is relatively short, less than 60 base pairs (bp), this limits the number of CpG sites that can be assessed in one assay.

3.2.1.1 *Bisulphite conversion*

In order to assess the DNA methylation of a target CpG site when using PCR-based methods, the DNA must first undergo bisulphite conversion.

Without bisulphite conversion, during PCR amplification, methylated cytosines are replaced by unmethylated cytosine (He and Cole, 2015), and therefore the methylation is lost after the first cycle of PCR.

Bisulphite conversion is the deamination of unmethylated cytosine to uracil. This reaction does not occur if the cytosine is methylated. Hence after bisulphite conversion the only cytosine remaining in the DNA is methylated cytosine. During PCR these methylated cytosines are converted to unmethylated cytosine and the uracil is converted to thymine. This preserves the methylation information of the original DNA sequence after PCR amplification (Grunau, Clark and Rosenthal, 2001).

The original method for bisulphite conversion was proposed by Frommer et al. (1992). Briefly, precipitated DNA is incubated with sodium bisulphite hydroquinone at pH5 & 50^{o}C for 16 or 40 hours, after which remaining bisulphite is removed via dialysis and the resulting solution is neutralized, desalted and re-suspended in storage solution and is ready for PCR (Frommer *et al.*, 1992). The harsh conditions of this original method have been shown to degrade the DNA during treatment. New commercial kits are not as problematic as several chemical improvements have been made to the reaction and therefore there is improved recovery. Hernández et al. (2013) compares the commercially available kits. The most commonly used kits in research are EpiTect^{TM} Bisulphite kit produced by Qiagen (Qiagen N.V. and its subsidiaries), or EZ DNA methylation Gold^{TM} kit produced by Zymo Research.

3.2.2 Tissue Selection

In DNA methylation research, the tissue of interest must be carefully chosen as more variability is observed between tissues from the same individual, than within tissue between individuals (Kochanek *et al.*, 1990). The ability to obtain tissue from metabolically relevant tissues within humans, such as muscle, liver and adipose tissue can be problematic in healthy individuals that have not required surgery. One key tissue used in genetics research, due to the less invasive collection method, is whole blood. This source of DNA is most beneficial when looking for genetic polymorphisms. Other cells used for this line of work include buccal epithelial cells and hair. However, it is not going to be relevant to use hair follicles or buccal swabs for many epigenetic studies where epigenetic signatures are specific to cell type. For example, when considering the role of DNA methylation in gene regulation, there is greater variance between tissues in the same individual than that observed between individuals within a single tissue (Eckhardt *et al.*, 2006; Byun *et al.*, 2009; Davies *et al.*, 2012). Consideration must also be given for samples derived from multiple cell types that have the potential for different methylation patterns (Siegmund and Laird, 2002; Wu *et al.*, 2011). The implication when designing a research study investigating epigenetic modifications is that the samples studied must be from the same tissue and chosen so that any changes that are observed in DNA methylation in a specific cell type are biologically relevant to the research question. In addition to epigenetic signatures depending on the type of cell, they are also influenced by the metabolic processes within the cell and the environment the cell is exposed to.

In regards to inflammation, within the blood are white blood cells that are important in systemic function and inflammation (Afman and Müller, 2012). These cells contain DNA that can be easily extracted from small volumes of blood. PBMCs isolated from blood are a heterogeneous mix of white blood cells and therefore will have varying epigenetic profiles. It is also important to consider that the blood composition, leukocyte composition of the whole blood was found to correlate with DNA methylation (Lam *et al.*, 2012), an issue that may influence associations between environmental factors and DNA methylation when using blood samples. Therefore, composition of whole blood should be determined for each individual and used in statistical models to correct the methylation values or complete the DNA methylation analysis on specific

blood cells types. Differences in DNA methylation in PBMCs have also been associated with demographic factors such as sex, age and ethnicity (Lam *et al.*, 2012). In this thesis all blood samples have had the cell composition analysed on a Beckman Coulter Counter (Beckman, USA) to allow for appropriate adjustments to be made for the methylation values. In this thesis, the method presented by Jones *et al.*, (2015) has been used to adjust DNA methylation values to account for the white blood cell composition. This method uses a sum of the mean methylation for the site and the unstandardised residual from a linear regression between DNA methylation (dependent variable) and the individual white blood cell counts (independent variables) to give an adjusted methylation value.

3.3 Target Identification and Justification

The overarching aim of this research is to investigate how ω-3 PUFAs and other fatty acids within the blood interact with inflammation, thus a critical component of this work was the identification of target genes and specific CpG sites within them. The current research into the interaction between fatty acids and DNA methylation, described in Chapter 1, has particularly focused on fatty acid metabolism genes and genome-wide methylation analysis. The methylation genome-wide association studies (meth-GWAS) research has identified gene pathways enriched for DMRs which are linked to fatty acids and include pathways involved in initiating and regulating inflammation. Therefore, the decision was made to investigate genes that have been associated with fatty acids or where their transcripts have been altered by fatty acid consumption.

Inflammatory diseases are often characterised with chronic activation of NFκB transcription factor and release of inflammatory cytokines (Epstein, Barnes and Karin, 1997) and therefore an important target for changes in DNA methylation by ω-3 PUFAs. Associations between dietary fats and DNA methylation in the NFκB pathway, measured using the 450k array, were observed in the Greek preadolescent cross-sectional cohort (Voisin *et al.*, 2015), and therefore may provide valuable targets within intervention studies. The transcription factor NFκB is a protein complex encoded by multiple genes including *NFKB1* and *NFKB2*. Furthermore, methylation of these genes have been associated with dietary intake of milk post-exercise and to exercise

interventions (Y. Zhang *et al.*, 2015; Masuki *et al.*, 2017). These two genes warrant further investigation as to their interactions with fatty acids.

Considering the cytokines within the NFκB pathways, the inflammatory cytokine tumor necrosis factor alpha (TNFα), encoded by the *TNF* gene, primarily mediates the acute-phase response in the inflammatory process (Sullivan *et al.*, 2007). TNFα is associated with a wide range of inflammatory disorders, and therefore is regularly a therapeutic target with anti-TNFα drugs (Bazzani *et al.*, 2009). When a macrophage cell line has been stimulated with LPS *in vitro*, TNFα secretion is decreased by the treatment of ω-3 PUFAs EPA and DHA (Mullen, Loscher and Roche, 2010). EPA treatment does not affect the stability of *TNF* mRNA in cytoplasm and therefore reductions in *TNF* mRNA as a result of EPA treatment is likely to be the result of decreased formation rather than degradation of the cytokine (Zhao *et al.*, 2004). The influence of ω-3 PUFAs on *TNF* expression may be the result of alterations in epigenetic mechanisms such as DNA methylation. Epigenetic regulation plays an important role in the control of *TNF* expression (Kochanek *et al.*, 1991; Takei *et al.*, 1996; Sullivan *et al.*, 2007). In a cell line that does not produce TNFα in response to LPS or phorbol myristate acetate (PMA), the promotor and exon-1 of *TNF* had higher methylation levels than TNFα expressing cells (THP-1 macrophages) which had a demethylated promotor and exon-1 (Sullivan *et al.*, 2007). Specifically, *TNF* has been shown to be hypomethylated in Crohn's disease patients (Nimmo *et al.*, 2012), and that CpG methylation interacts with histone methylation to silence *TNF* expression during endotoxin tolerance (Gazzar *et al.*, 2008). Although some previous research has not observed associations between DNA methylation of *TNF* and fatty acids (Lee *et al.*, 2013; Bollati *et al.*, 2014); other research has found environmental factors, including diet and fatty acids, that do have relationships with *TNF* methylation (Campión *et al.*, 2009; Hermsdorff *et al.*, 2013; Bollati *et al.*, 2014; Gómez-Uriz *et al.*, 2014). For this reason, it is worth considering cytokine genes as potential targets for further investigation.

The expression of *IL6* is also decreased after the treatment of ω-3 Fatty acids (Cawood *et al.*, 2010). There is previous evidence for the regulation of gene expression through alterations in the methylation of the *IL6* promotor (Poplutz *et al.*, 2014). The occurrence of DNA methylation of four CpG sites in the *IL6* promoter is

significantly higher in cancer cell lines and associated with lower IL6 mRNA and methylation of the *IL6* promoter was inversely related to mRNA levels (Tekpli *et al.*, 2013). In addition, when macrophages are stimulated with LPS, increased mRNA is associated with lower *IL6* promotor methylation (Nile *et al.*, 2008). Measures of adiposity have been found to associate with *IL6* promotor methylation (Na *et al.*, 2015). A cross sectional study comparing ω-3 PUFA in erythrocyte membranes with *IL6* methylation of a single CpG site within the *IL6* gene found that DHA and total ω-3 fatty acid was negatively associated with the methylation level. Furthermore, methylation of this site was correlated with IL6 expression and plasma IL6 concentration (Ma *et al.*, 2016). A separate CpG site in the same study found no relationship between IL6 expression and plasma IL6 concentration (Ma *et al.*, 2016), indicating the importance of site selection. In addition, this study used 450k array measurements of the methylation at each of these sites, demonstrating that although 450k arrays sample a high number of sites, it depends if those individual CpGs are biologically relevant to be altered or have associations with fatty acids.

A third cytokine, for which evidence of alteration by of ω-3 PUFAs exists, is IL1β (Allam-Ndoul *et al.*, 2016). There is also evidence of transcriptional regulation of this cytokine through methylation of the promotor (Hashimoto *et al.*, 2009; Aoi *et al.*, 2011; Tekpli *et al.*, 2013). In cancer cells, CpG sites near the transcription start site (TSS) of *IL1B* “interleukin 1 beta” promoter were significantly more methylated and associated with lower IL1B mRNA levels (Tekpli *et al.*, 2013). Methylation of the *IL1B* promoter was inversely related to mRNA levels in cultured human lung cells (Tekpli *et al.*, 2013). Two CpG sites (-299 and -256bp from Exon 1) in the *IL1B* promoter were differentially methylated between control samples and cytokine-treated chondrocytes (Hashimoto *et al.*, 2009). There is currently no evidence on the effects of ω-3 PUFAs on the methylation levels of IL1B and therefore will investigate this further in this thesis.

The following genes were considered for assay design: *NFKB1*, *NFKB2* (failed assay design), *TNF*, *IL6* and *IL1B*.

3.4 Pyrosequencing Assay Design

3.4.1 Assay Design

Approximately half of all gene promotors contain CpG islands, the methylation of these islands interacts with other epigenetic controls such as histones to control the transcription of the gene (Deaton and Bird, 2011). Methylation in promotors and regions proximal to promotors which do not contain CpG islands has also been shown to regulate gene expression (Jones, 2012). This has been observed in inflammatory genes including *NOS2 "*nitric oxide synthase 2", *IL2 "*interleukin 2", *IFNG*, *MMP13* "Matrix Metallopeptidase 13", *IL1B* , and *TNF*; (Northrop *et al.*, 2006; Gazzar *et al.*, 2008; Berkley *et al.*, 2013; Hashimoto *et al.*, 2013; Williams *et al.*, 2013; Gross *et al.*, 2014). Additionally, it is not only the DNA methylation that occurs in the promotor region near the TSS that plays an important role in regulation of gene expression, with evidence suggesting that 5mC in gene bodies and non-promotor regions plays an important role (Lou *et al.*, 2014). Methylation in gene bodies alters the activity of the gene by preventing the binding of transcription proteins (Choy *et al.*, 2010). There is also evidence that methyl-CpG-binding proteins bind to methylated DNA and recruit other proteins, such as histone deacetylases, which alter the chromatin structure which reduces transcription (Nan *et al.*, 1998). However there are also recent reports that methylation can also promote transcription of a gene through the recruitment of protein complexes (Harris *et al.*, 2018), thus aiding the fine tuning of gene expression through DNA methylation.

Careful considerations must be taken when designing assays to measure the DNA methylation of candidate genes. In order to design assays that were within biologically relevant regions of each of the target genes, the current literature was used to pick regions of the genes which had either shown some associations with fatty acids (where available) or where there was previous evidence of transcriptional activation of the gene. The FASTA DNA sequence for each target gene was downloaded from Ensemble (EMBL-EBI) NCBI build 38 and the sequence for the target region within the gene was imported into the PyroMark™ assay design software (Qiagen, Germany). The bisulphite conversion of the sequence is performed *in silico* by the assay design software. Manual selection of the target region within the bisulphite-

converted target region was completed prior to running the software to generate potential primer sets. The PyroMark™ software scored each of the assays generated out of 100. For each target region the highest scoring assay was assessed for suitability and manually adjusted if required.

PCR Primers should have the following characteristics where possible (Chuang, Cheng and Yang, 2013):

- Primer length between 20-32 nucleotides
- No CpG sites within the binding region of the primer
- The GC content <60%
- Annealing temperature below 65°C
- Sequences of homopolymers and repetitive sequences should be avoided
- No self-complementarity or secondary structures
- Amplification product should be <350bp

The specificity of the PCR primer sets was assessed using online primer design tools:

- *Bisearch* (http://bisearch.enzim.hu/?m=genompsearch) primer search function run the PCR *in silico* using the bisulphite converted DNA of the selected organism. This is used to identify the number of products that will be formed by the PCR primers.
- *PrimerBLAST* (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) checks the primers against the reference assembly for human genomic DNA, this is used to confirm that non-converted DNA will not be amplified by the primer sets.

The assays designed are detailed in Table 3-1.

Table 3-1 Pyrosequencing assays

Assay	Primer Sequence		Assay Score (PyroMark)	Number of CpG sites covered	Validation	Accuracy (R^2)	Coefficient of Variation (%)
Assay A (***TNF* Promotor**)	Forward PCR Reverse PCR Sequencing	5'- biotin-TGTGAGGGGTATTTTTGATGT-3' 5'-CCTTAATAAAAAAACCCATAAACTCATC-3' 5'-AAACCCATAAACTCATCTA-3'	75	5	Failed	N/A	N/A
Assay B (***TNF* exon 1**)	Forward PCR Reverse PCR Sequencing	5'-GGAAAGGATATTATGAGTATTGAAAGTATG-3' 5'-biotin-CTAAAACCCCCCTATCTTCTTAAA-3' 5'-ATTATGAGTATTGAAAGTATGAT-3'	100	4	Passed	0.9915	7.84
Assay C (***IL6* -1099**)	Forward PCR Reverse PCR Sequencing	5'-GGGAAGAGGGTTTTTGAATTAG-3' 5'-biotin-CTCCCTCTCCCTATAAATCTTAATTTAA-3' 5'-TTGAATTAGTTTGATTTAATAAGAA-3'	92	6	Passed	0.9939	0.38
Assay D (***IL6* -666**)	Forward PCR Reverse PCR Sequencing	TGGGTTGAAGTAGGTGAAGAAA-3' 5'-biotin-ACTACCTAACCATCCTCAAATT-3' AGGTGAAGAAAGTGG-3'	95	4	Passed – very low methylation levels on samples	N/A	N/A
Assay E (***IL1B* -299**)	Forward PCR Reverse PCR Sequencing	5'-ATGGAAGGGTAAGGAGTAGTAA-3' 5'-biotin-ATATCTTCCACTTTATCCCACATATAC-3' 5'-ATGTAAATATGTATTGTTTTTTTGA-3'	89	2	Failed	N/A	N/A
Assay F (***IL1B* -1063**)	Forward PCR Reverse PCR Sequencing	5'-GGGAGGTTGAGGTAGGAGAAT-3' 5'-biotin-CACCCTCCTTTACCTCTTTAACAAATCTA-3' 5'-GGAGAATGGTGTGAA-3'	92	5	Passed	0.9934	0.97
Assay G (***NFKB1*)**	Forward PCR Reverse PCR Sequencing	5'-GGTTGGTTGAGTTAGTTTAGAGTTAAA-3' 5'-biotin-CTCAAAACCCAAAAAACCCCTTCTC-3' 5'-GTTAGTTTAGAGTTAAATTTTTAGT-3'	89	2	Passed – very low methylation levels on samples	N/A	N/A

3.4.2 Tumor Necrosis Factor (TNF) Assay

The primers were designed to amplify two regions within the gene encoding TNFα which had been demonstrated to either change with an inflammatory stimulus or associated with expression. The FASTA sequence (GRCh 38: 6: 31574967: 31578936: 1) for the *TNF* gene was used to design the assays for two regions of the gene: promotor and exon 1, refer to Figure 3-2. Assay A was designed to measure the methylation of five CpG sites within the promotor of *TNF* (-170, -164, -162, -147 and -120 bp from the TSS). The highest score for an assay in this region was 75; this was the result of a homopolymer sequence adjacent to the CpG sites that were being analysed. Assay B targeted four CpG sites within exon 1 of *TNF* (+197, +202, +214 and +222 bp from the TSS), this assay scored the maximum with a score of 100.

Bisearch confirmed that PCR product would be formed from the correct regions on the sense strand of chromosome 6. The primers were then run through *PrimerBLAST*, for Assay A (Promotor) there were no targets found within the reference assembly. Assay B (Exon 1) found two potential products, however it was considered that these products would not interfere with the assay due to the product length (>1000bp) and confirmed during validation of the assay via gel electrophoresis and assay performance.

3.4.3 Interleukin 6 (IL6) Assay

With regards to the *IL6* gene, two regions were identified in the gene promotor which had associated with fatty acids and transcription of the gene. The FASTA sequence (GRCh38:7:22725284:22732602:1) was used for the *IL6* assay design. Assay C was designed to cover the CpG site -1099 from the TSS and Assay D covered CpG site -666 from the TSS. Both assays scored well on the assay design software with 95 and 92 respectively. Using *Bisearch*, one PCR product was found for each of the assays designed and *PrimerBLAST* did not identify any products from non-bisulphite converted DNA, indicating only bisulphite converted DNA would amplify.

3.4.4 Interleukin 1 Beta (IL1B) Assay

The FASTA sequence (GRCh38:2:112829151:112837503:-1) was used for the *IL1B* assay design. The *IL1B* assay was designed on the reverse strand of the gene with Assay E covering the CpG -299bp from the TSS and Assay F covering -1099bp from the TSS. These assays scored 89 and 92 respectively.

3.4.5 Assay validation

Once the assays had been designed for the regions of interest, the assays were ordered from the custom assay service provided by Qiagen. Validation of the assays was completed by using the PyroMark® PCR kit (Qiagen, Germany) in a Veriti® thermocycler (Applied Biosystems Inc., USA) for amplification of target DNA. The PCR product was sequenced with the PyroMark® Q48 Autoprep (Qiagen, Germany) using Advanced CpG Reagents (Qiagen, Germany) in accordance with the manufacturer's protocols.

The specificity of the primers to detect methylated and unmethylated cytosines was evaluated using EpiTect Control DNA (Qiagen, Germany), then mixed to varying methylation percentages. In addition, interaction between the primers for loop formation was examined using a specific set of control experiments:

- PCR negative (no template DNA),
- PCR negative (no template DNA, no sequencing primer),
- Sequencing primer (no PCR product),
- PCR product (non-converted template DNA).

The performance of each assay was confirmed by running methylated and unmethylated bisulphite-converted DNA (Qiagen, Germany), a combination of the two bisulphite-converted DNA controls (50% methylated), unmethylated non-converted DNA (uncon) and negative no template control (NTC). Two additional reactions of 50% methylated control DNA were included to allow for a no sequencing primer (noseq) control. PCR product was visualised on a 2% agarose gel stained with SybrSafe, an example gel is shown in Figure 3-1.

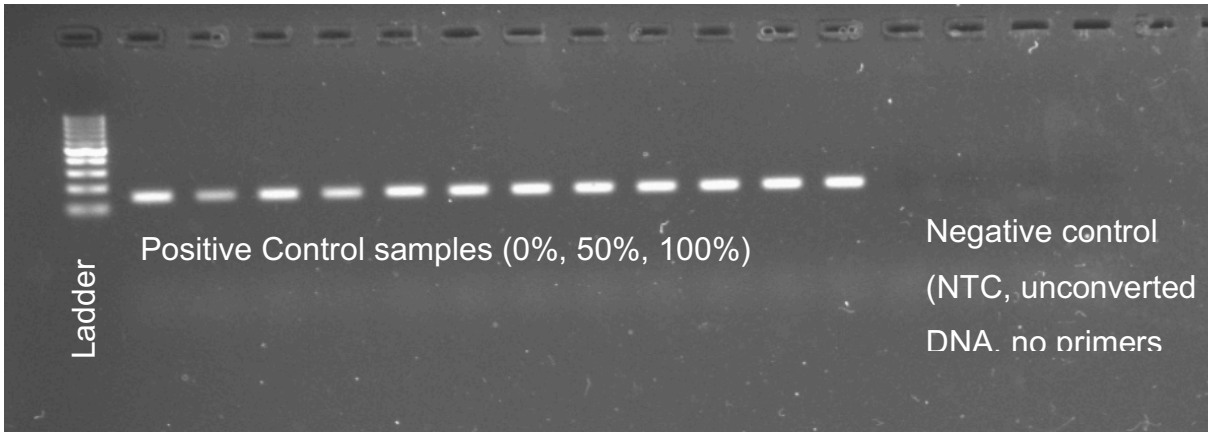

Figure 3-1 Gel electrophoresis image of the IL1B validation. No template control (NTC)

PCR-based methods of methylation analysis, such as pyrosequencing can be affected by PCR bias, because the methylated and unmethylated DNA amplify at different efficiencies. In order to check each of the designed assays for PCR biases, known quantities of methylated and unmethylated control DNA (Qiagen, Germay) were mixed at incremental percentages to produce standard curves.

The bisulphite-converted DNA was amplified using custom designed assays with the PyroMark® PCR kit (Qiagen, Germany) in a Veriti® thermocycler (Applied Biosystems Inc., USA). PCR product was used for CpG quantification with the PyroMark® Q48 Autoprep (Qiagen, Germany) using Advanced CpG Reagents (Qiagen, Germany) in accordance with the manufacturers protocol. The assay covered the methylation sites +197, +202, +214 and +222 bp from the transcription start site of *TNF*. The percentage methylation of the four CpG sites was calculated within the software (PyroMark® Q48 Autoprep 2.4.1 Software, Qiagen, Germany) and the methylation percentages were exported for further analysis.

Three assays passed validation and had methylation levels from blood samples of >5%, refer to Table 3-1. These assays *TNF* (Assay B), *IL6* (Assay C) and *IL1B* (Assay F) are used throughout this thesis, the genomic location of these assays is shown in Figure 3-2, Figure 3-3 and Figure 3-4.

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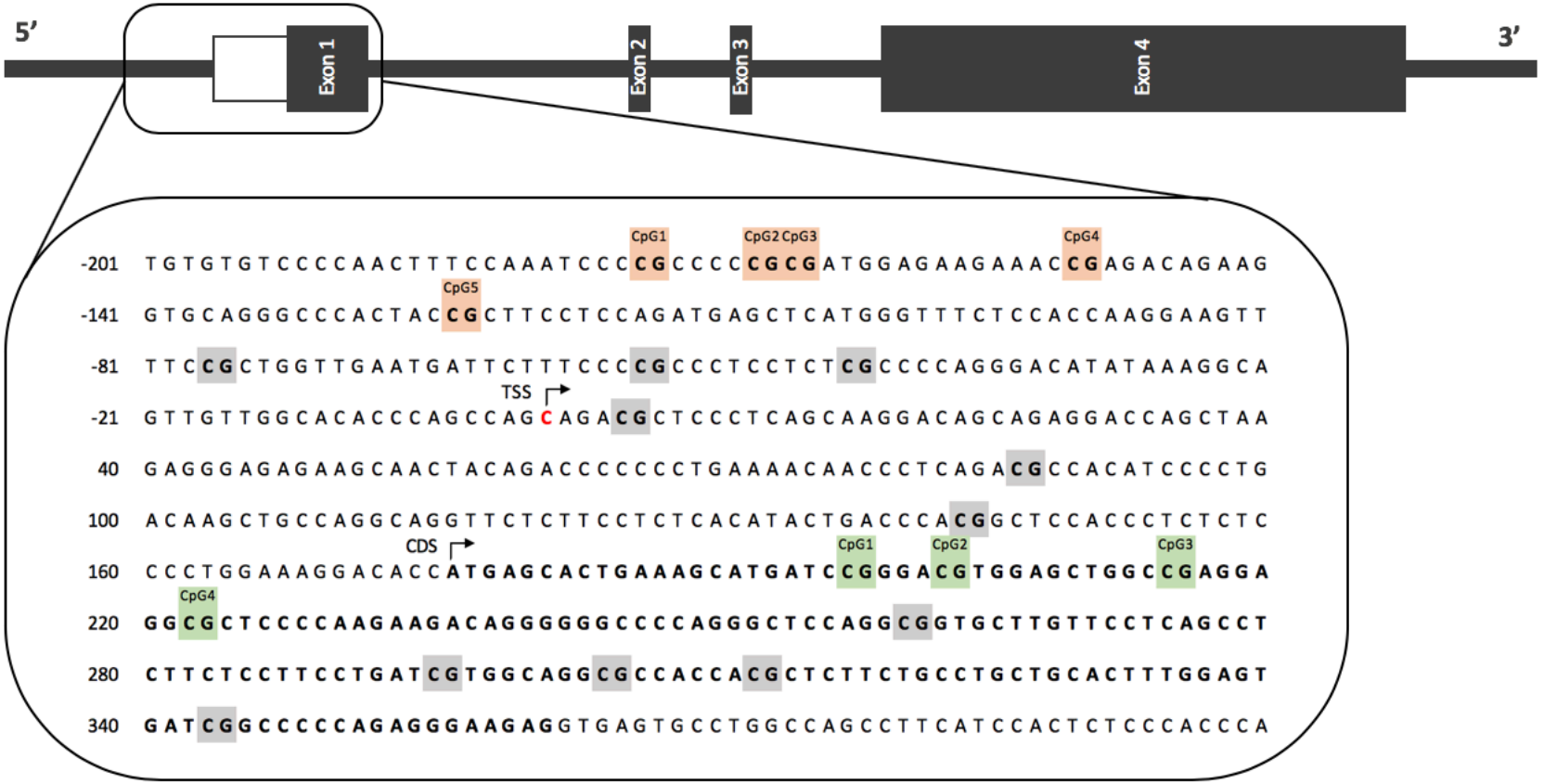

Figure 3-2 Genomic structure of TNF (MIM 191160; GRCh 38: 6: 31574967: 31578936: 1). Sequence shown for promotor and exon 1 with Cytosine-Guanine dinucleotides (CpG) sites indicated. Assay A (red) covers five CpG sites CpG1 (-170), CpG2 (-164), CpG3 (-162), CpG4 (-147) and CpG5 (-120) base pairs from the Transcription Start Site (TSS). Assay B (green) covers four CpG sites CpG1 (+197), CpG2 (+202), CpG3 (+214) and CpG4 (+222) base pairs from the TSS. Coding DNA Sequence (CDS). Exon 1 displayed in bold.

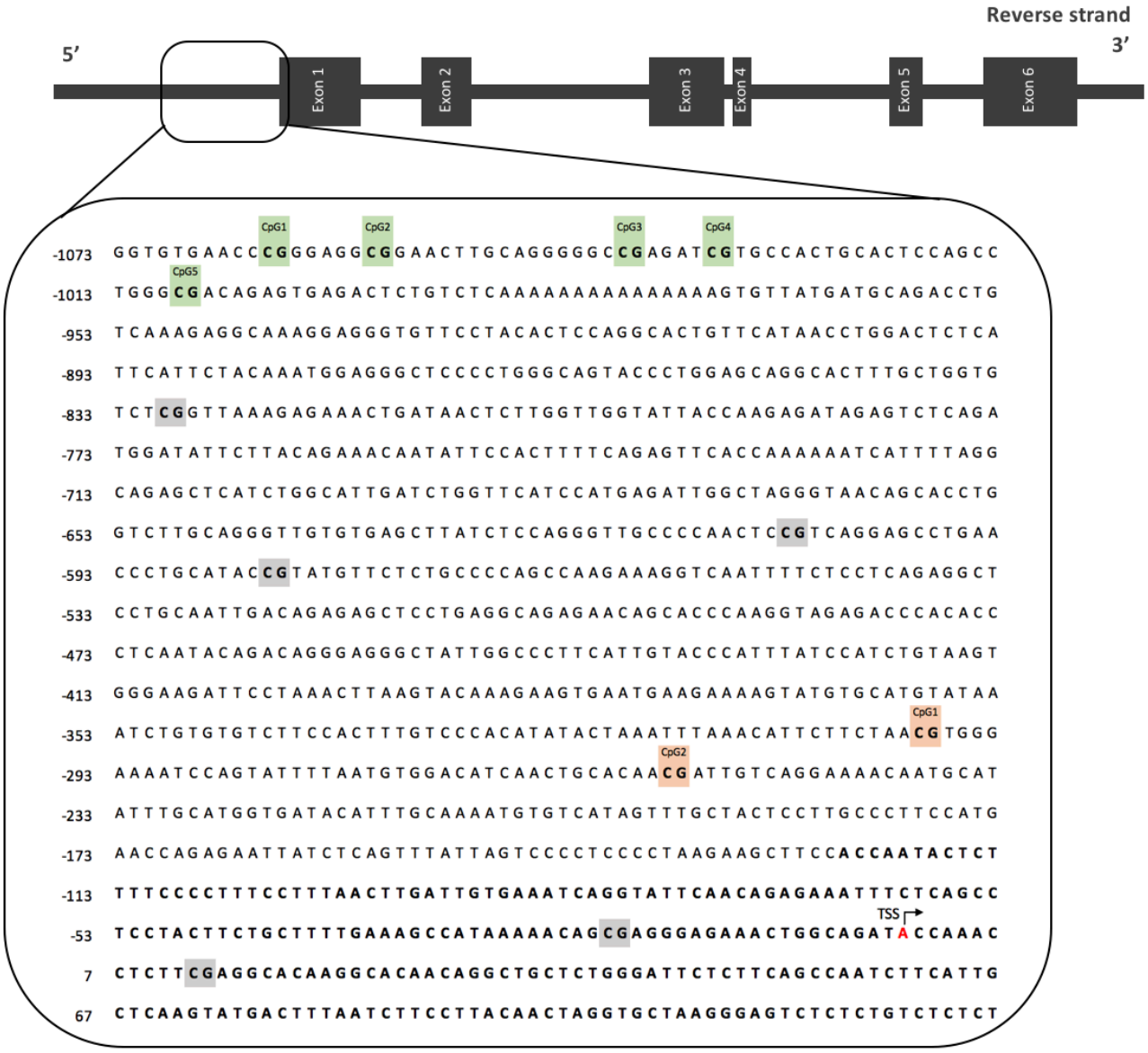

Figure 3-3 Genomic structure of IL1B (MIM 147720; GRCh38:2:112829151:112837503:-1). Sequence shown for promotor and exon 1 with Cytosine-Guanine dinucleotides (CpG) sites indicated. Assay F (red) covers five CpG sites CpG1 (-1063), CpG2 (-1056), CpG3 (-1039), CpG4 (-1033) and CpG5 (-1009) base pairs from the Transcription Start Site (TSS) Assay E (green) covers two CpG sites CpG1 (-299), and CpG2 (-256) base pairs from the TSS. The start of Exon 1 displayed in bold.

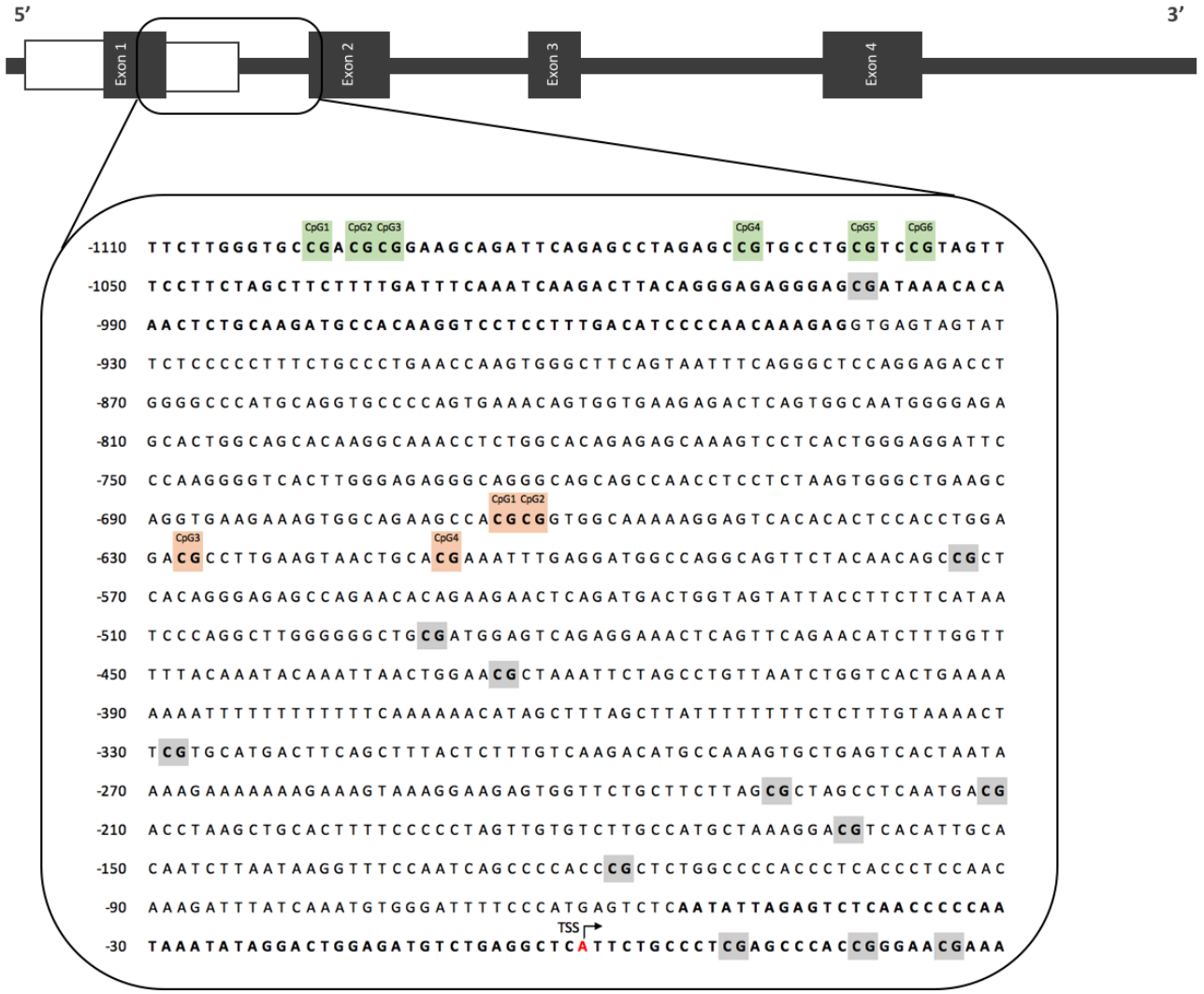

Figure 3-4 Genomic structure of IL6 (MIM 147620; GRCh38:7:22725284:22732602:1). Sequence shown for promotor with Cytosine-Guanine dinucleotides (CpG) sites indicated. Assay D (red) covers four CpG sites CpG1 (-666), CpG2 (-664), CpG3 (-628) and CpG4 (-610) base pairs from the Transcription Start Site (TSS) Assay C (green) covers six CpG sites CpG1 (-1099), CpG2 (-1065), CpG3 (-1094), CpG4 (-1069), CpG5 (-1061) and CpG6 (-1057) base pairs from the TSS. Exons within the region are displayed in bold.

Chapter 4 Cross sectional analysis of *TNF* methylation and associations with blood fatty acid profile [1]

4.1 Introduction

The global trend towards the Western diet has altered the quantity and composition of dietary fat (Popkin, 2006). This change is significant because evidence now indicates that consuming the right sort of fat is important (de Oliveira Otto *et al.*, 2012). Dietary fatty acids, SFAs, MUFAs, ω-3 PUFAs and ω-6 PUFAs are reported as having differing effects upon health (Estruch *et al.*, 2006; Lunn, 2007; Mozaffarian, Micha and Wallace, 2010; Innes and Calder, 2018). Some of the reported effects are beneficial whilst others are detrimental. For example, research suggests that the replacement of SFAs with PUFAs is associated with lower incidence of CHD (Mozaffarian, Micha and Wallace, 2010). Furthermore, consuming MUFAs rather than SFAs has a protective effect for CHD, diabetes and cancer (Lunn, 2007).

The nutritional components of diet, including fatty acids, ultimately contribute to cell environment. Cells respond to their environment through changes in epigenetic mechanisms, which include histone modifications, microRNAs, chromatin remodelling and DNA methylation (Joseph, Abey and Henderson, 2016). Some of these responses result in alterations to and errors within the epigenome and are implicated in diseases such as cancer, CHD, and inflammatory bowel disease (Jones and Baylin, 2007; Barnett *et al.*, 2010; Muka *et al.*, 2016). A common component of these diseases is chronic inflammation (Chen *et al.*, 2018). Fatty acids are involved with the inflammatory process on multiple levels, including the production of eicosanoids,

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[1] Adapted from **Hussey B.,** Steel R., Taylor I.M., Gyimah B., Reynolds J., Lindley M.R., & Mastana S.S. (In review) DNA methylation of tumor necrosis factor (*TNF*) alpha gene is associated with specific blood fatty acid levels. Epigenetics.

alterations in cell signalling and interactions with gene expression, amongst others (Calder, 2012a). It is the epigenetic interaction of fatty acids, specifically with regards to DNA methylation, which will be examined using a cross sectional study design in this chapter.

Interactions between fatty acids and methylation have been the subject of research utilising both cross-sectional loci-specific and meth-GWAS. Relationships between dietary fatty acids and biologically significant pathways related to inflammation have been observed (Hermsdorff *et al.*, 2013; Aslibekyan *et al.*, 2014; Haghighi *et al.*, 2015; Voisin *et al.*, 2015; Cui *et al.*, 2016; Ma *et al.*, 2016; Rahbar *et al.*, 2018). In a meth-GWAS study of an adolescent population, fatty acid ratios (including (MUFA+PUFA)/SFA and PUFA/SFA) in the diet showed significant associations with pathways linked to nuclear factor kappa B (NFκB), peroxisome proliferator-activated receptor alpha (PPARα), LEP and IL6 (Voisin *et al.*, 2015). Using a 24-hour dietary recall, the authors were only capturing the short-term dietary effects of the fatty acids. By measuring the biochemical ω-3 PUFA status of blood, a separate meth-GWAS study considered the longer-term dietary habits, incorporation into the blood and the endogenous production of fatty acids (Aslibekyan *et al.*, 2014). This research of an Alaskan (Yup'ik) cohort found 27 significant DMRs when splitting the cohort by ω-3 PUFA status; DMRs were found in genes which are implicated in disease and inflammation including helicase-like transcription factor (*HLTF*), actin a 2 smooth muscle, fas cell surface death receptor (*FAS*), neuron navigator 1, chemokine ligand 17, and aryl-hydrocarbon receptor repressor (Aslibekyan *et al.*, 2014). Additionally, it was found that the majority of sites (78%) had increased methylation with higher ω-3 status, suggesting that increased levels of ω-3 PUFA may improve epigenomic stability.

In candidate gene approaches, associations have been investigated between dietary fatty acids and the DNA methylation of genes involved in fatty acid metabolism. The methylation of the *FADS1*, and *FADS2* genes were investigated in three cohorts, where negative associations with ω-6 PUFAs were observed in all cases (Cui *et al.*, 2016; Rahbar *et al.*, 2018). The methylation of these genes, and fatty acid metabolism gene elongation of very long chain fatty acids protein 5 (*ELOVL5*), has been associated with clinical outcomes such as major depressive disorder (Haghighi *et al.*,

2015). In addition, associations between PUFA's and suicide risk were explained by *ELOVL5* methylation (Haghighi *et al.*, 2015). Genes involved with inflammation have briefly been studied. A CpG site within the *IL6* gene showed a negative association with total ω-3 PUFA, with dependency on a single nucleotide polymorphism within the same gene (Ma *et al.*, 2016).

The interactions that have been discussed so far, suggest that there is scope to investigate further the relationship between fatty acids and methylation focusing on genes associated with the inflammatory process. Chronic inflammatory diseases are often characterised by elevated levels of circulating inflammatory cytokines, such as TNFα. The research, which is the subject of this chapter, has specifically focused on the effect of methylation on this gene.

TNFα is produced predominantly in monocytes and macrophages; it is an important mediator in inflammation and in the regulation of multiple other genes, including but not limited to: transcription factors, cytokines, growth factors, receptors, cell adhesion molecules and inflammatory mediators (Vilček and Lee, 1991; Locksley, Killeen and Lenardo, 2001; Bradley, 2008). Transcriptional control of the *TNF* gene occurs partly through epigenetic mechanisms (Sullivan *et al.*, 2007; Gazzar *et al.*, 2008; Campión *et al.*, 2009; Falvo, Tsytsykova and Goldfeld, 2010; Cordero *et al.*, 2011; Gowers *et al.*, 2011). Furthermore, DNA methylation levels within the promotor and exon 1 regions of the *TNF* gene are associated with plasma TNFα cytokine levels (Hermsdorff *et al.*, 2013). The DNA methylation profile of *TNF* has been shown to be associated with diseases including Crohn's disease, post-traumatic stress disorder and age-related inflammation, as well as truncal fat and diet (Gazzar *et al.*, 2008; Pieper *et al.*, 2008; Campión *et al.*, 2009; Cordero *et al.*, 2011; Gowers *et al.*, 2011; Nimmo *et al.*, 2012). Using a targeted candidate gene approach, this research has sought to establish whether there is a relationship between blood fatty acid levels and the DNA methylation profile within the first exon of the *TNF* gene.

4.2 Method

4.2.1 Participants

Ethical clearance was granted from the Loughborough University Ethics Approvals (Human Participants) Sub-Committee (reference R16-P074) and all participants provided written informed consent. Adults (n=88, 18+ years) were recruited from the local community in the Loughborough Town area. Participants were free of blood borne viruses; there were no other exclusion criteria.

4.2.2 Study visit

Participants came to the National Centre for Sport and Exercise Medicine at Loughborough University for one visit. After obtaining written consent, anthropometric measurements of height and weight were recorded; two participants refused measurement of height and weight and therefore were not included in analysis requiring these variables. A finger prick capillary blood sample was collected into three 300µL EDTA-coated microvette tubes (Microvette® CB 300 µl, K2 EDTA, Sarstedt, Germany) for blood cell count and DNA extraction. Four dried blood spots (DBS) were collected for fatty acid analysis.

4.2.3 Fatty acid measurements

DBSs were collected on Whatman cards (903 Protein saver, Sigma-Aldrich) pre-treated with butylated hydroxytoluene (supplied by the Loughborough University Chemistry department) to stabilise the blood fatty acids for up to eight weeks at room temperature (Metherel *et al.*, 2013). Each DBS was punched from the card, weighed and frozen at -80^{o}C within eight weeks of collection. Gas chromatography mass spectrometry (GC-MS), carried out by Boakye Gyimah in the Loughborough University Chemistry department, was used to analyse the fatty acid composition of the DBS. Methyl esters were prepared using the method from Ichihara and Fukubayashi, (2010). Briefly, one of the 30mm^2 punched samples was derivatised by acetyl chloride (99%, Sigma Aldrich) and methanol (HPLC grade, Fisher Scientific) at 70^{o}C for 60 minutes to produce fatty acid methyl esters (FAMEs) and reconstituted in 100µl hexane (HPLC grade, Fisher Scientific) containing 10µg/ml of internal standard

(methyl heptadecanoate, analytical standard 51633-1G, Sigma Aldrich). The sample was analysed by GC-MS, FAMEs were characterized by electron ionization in positive ion full scan mode. Fatty acid quantities were determined based on the relative abundance to the internal standard. Fatty acids are presented as their percentages of total fatty acids (%TFA.).

4.2.4 DNA extraction and bisulphite conversion

One aliquot of the capillary blood was used for a cell count (Beckman Coulter Counter, USA). Aliquots of capillary blood were frozen at -80ºC until analysis. DNA was extracted from the capillary blood and bisulphite-converted using EpiTect™ Fast LyseAll Bisulphite conversion kit (Qiagen, Germany) using the manufacture's protocol. Briefly, the blood sample was lysed, and proteins denatured, the resulting pellet was re-suspended in PBS and added directly into the bisulphite reaction. The DNA was sodium bisulphite-treated and subjected to two cycles of denaturing at 95ºC for 5 minutes and incubation 60ºC for 20 minutes. Bisulphite-converted DNA was desulfonated and purified using MinElute spin columns provided. Successful bisulphite conversion was assessed using a bisulphite-converted DNA-specific dispensation during the pyrosequencing, as displayed on a pyrogram in Figure 4-1 highlighted in orange.

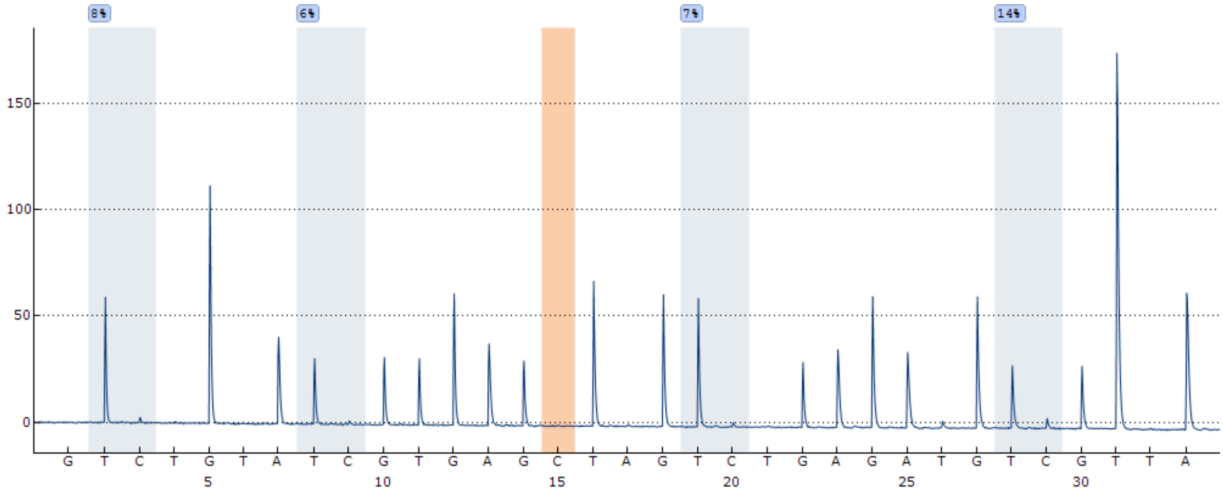


Figure 4-1 Example pyrogram for TNF exon 1 assay covering Cytosine-Guanine dinucleotides (CpG) sites +197, +202, +214 and +222 from the transcription start site, highlighted grey. Dispensation 15, highlighted in orange, served as a bisulphite conversion control.

4.2.5 DNA methylation analysis

The methylation of four CpG sites within Exon 1 of the *TNF* were measured using bisulphite pyrosequencing (PyroMark® Q48 Autoprep System, Qiagen, Germany). Primers, designed in Chapter 3, are provided in Table 4-1. The extracted bisulphite-converted DNA was amplified using the PyroMark® PCR kit (Qiagen, Germany) in a Veriti® thermocycler (Applied Biosystems Inc., USA). PCR product was used for CpG quantification with the PyroMark® Q48 Autoprep (Qiagen, Germany) using Advanced CpG Reagents (Qiagen, Germany) in accordance with the manufacturers protocol. The assay covered the methylation sites +197, +202, +214 and +222 bp from the transcription start site of *TNF*. The percentage methylation of the four CpG sites was calculated within the software (PyroMark® Q48 Autoprep 2.4.1 Software, Qiagen, Germany) and the methylation percentages were exported for further analysis.

Table 4-1: Pyrosequencing primers for TNF (MIM 191160) exon 1. The assay covers four methylation sites +197, +202, +214 and +222 base pairs from the transcription start site.

Pyrosequencing Primer Sequence	
Forward PCR Primer	5'-GGAAAGGATATTATGAGTATTGAAAGTATG-3'
Reverse PCR Primer	5'-biotin-CTAAAACCCCCCTATCTTCTTAAA-3'
Sequencing Primer	5'-ATTATGAGTATTGAAAGTATGAT-3'

4.2.6 Statistical Analysis

All statistical analysis was performed using IBM SPSS statistics software (SPSS version 23). The data distribution was assessed for normality by Shapiro-Wilk's test ($p > 0.05$). Group differences between sex were assessed using an independent sample t-test for normally distributed data and a Man Whitney-U test where appropriate. Differences between sex for categorical data were assessed using chi-square.

A key consideration in DNA methylation studies is the composition of the white blood cells (WBCs) from which the DNA is extracted. The DNA methylation values are therefore presented raw and adjusted for the cell heterogeneity of the capillary blood. The method presented by Jones *et al.*, (2015) was used to adjust DNA methylation values to account for the white blood cell composition. In brief, the adjusted figure is a sum of the mean methylation for the site and the unstandardised residual from a linear regression between DNA methylation (dependent variable) and the individual white blood cell counts (independent variables).

Spearman's correlation analysis was used to assess the relationship between the methylation (raw and adjusted) percentage of the four sites studied and the fatty acids identified within the blood. A p-value lower than 0.05 was considered significant. Using an expected $\beta = 0.2$ and $r = 0.3$, sample size required was 85.

4.3 Results

Table 4-2: Demographic characteristics of the sample population

	All participants (n=88)	Males (n = 30)	Females (n = 58)	*p*
Age (years)[a.]	25 (22-41)	30 (23-47)	24 (21-39)	.210
Height (cm)[b,d.]	170.3 ± 8.7	177.3 ± 6.7	166.6 ± 7.2	**<.001**
Weight (kg)[a,d.]	70.2 (59.9-80.1)	80.6 (75.5-88.9)	63.3 (56.6-73.0)	**<.001**
BMI [a,d.]	23.9 (21.9-26.5)	24.9 (22.7-27.3)	22.9 (21.2-26.0)	.071
Current smoker (n, %)[c.]	7 (8.0%)	4 (13.3%)	3 (5.2%)	.180
Ever smoked (n, %)[c.]	24 (27.3%)	10 (33.3%)	14 (24.1%)	.359
Total white blood cells (10^9/L) [a.e.]	6.69 ± 1.40	6.19 ± 1.12	6.94 ± 1.47	**.018**
Neutrophils (10^9/L) [a.e.]	3.95 ± 1.09	3.61 ± 0.90	4.13 ± 1.15	**.039**
Lymphocyte (10^9/L) [a.e.]	1.99 ± 0.55	1.87 ± 0.33	2.06 ± 0.63	.132
Monocytes (10^9/L) [a.e.]	0.48 ± 0.12	0.47 ± 0.11	0.48 ± 0.12	.928
Eosinophils (10^9/L) [a.e.]	0.18 ± 0.11	0.15 ± 0.07	0.19 ± 0.12	.115
Basophils (10^9/L) [a.e.]	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	.808
Large immature cells (10^9/L) [a.e..]	0.04 ± 0.03	0.03 ± 0.02	0.04 ± 0.03	.181

a. Data was non-normally distributed, and data presented as median (lower quartile-upper quartile); differences between groups assessed by Man-Whitney-U difference test.
b. Data was normally distributed and presented as mean ± standard deviation; differences between groups assessed by independent samples t-test.
c. Categorical data, differences between groups assessed by Pearson chi-square
d. height and weight data missing for 2 female participants, (total n=86, female n=56)
e. Blood cell count data was not available for 3 participants (total n=85, males n=29, females n=56)

4.3.1 Sample demographic

Eighty-eight adults (Male n=30 and Female n=58) were recruited, details of the sample demographic are shown in Table 4-2. The age ranged from 18 to 74 years and there was a positive skew towards younger individuals, the sample contained 4 individuals above the age of 60 years. A small number of individuals were current smokers (n=7) within the sample, with almost a third (n=24) had considered themselves smokers at some point during their life. Height and weight differed between the sex, with male participants significantly taller and heavier than the female participants. In addition, a significant difference in total white blood cell count and neutrophil cell count was observed between males and females, therefore adjustment of DNA methylation values for cell composition was calculated both as a whole sample and separately split by sex.

4.3.2 DNA methylation

The average methylation of the four sites studied was 8.71%. DNA methylation was not significantly different between males and females, refer to Table 4-3. Moreover, this remained after adjustment for white blood cell composition (data not shown) despite the significant difference in white blood cell count between the sex.

Table 4-3 DNA methylation percentages for four Cytosine-Guanine dinucleotides (CpG) sites (+197, +202, +214 and +222 base pairs from transcription start site) located within exon 1 of the TNF gene (encoding cytokine Tumor Necrosis Factor alpha, TNFα)

DNA Methylation (%)	All (n=88)	Males (n = 30)	Females (n = 58)	p
CpG 1 (+197)	8.8 ± 2.3	8.6 ± 2.0	8.9 ± 2.4	.511
CpG 2 (+202)	6.5 ± 2.0	6.3 ± 2.1	6.6 ± 1.9	.568
CpG 3 (+214)	7.3 ± 2.1	7.2 ± 2.3	7.3 ± 2.0	.679
CpG 4 (+222)	12.3 ± 2.9	12.0 ± 3.2	12.4 ± 2.8	.520
CpG average	8.7 ± 2.1	8.5 ± 2.3	8.8 ± 2.1	.532

Data was normally distributed and presented as percentage methylation (%) mean ± standard deviation; differences between groups assessed by independent samples t-test. Cytosine-Guanine dinucleotides (CpG).

4.3.3 Fatty acid profile of peripheral blood

A total of 25 fatty acids were identified from the dried blood spots, presented in Table 4-5 as a percentage of total fatty acid. A ratio of 3.04 was observed for ω-6 to ω-3 PUFAs. There was no significant difference in fatty acid ratios between the sex, Table 4-4. The ω-3 PUFA DPA was significantly different between sex (p=0.05); all other fatty acids identified did not show any difference between the sexes, Table 4-5.

Table 4-4 Fatty acid ratios from dried blood spots

Fatty Acid Ratios	All (n=88)	Males (n = 30)	Females (n = 58)	*p*
MUFA / SFA	0.48 ± 0.08	0.47 ± 0.09	0.49 ± 0.08	0.199
PUFA / SFA	0.65 ± 0.10	0.64 ± 0.08	0.66 ± 0.10	0.482
(MUFA+PUFA) / SFA	1.13 ± 0.12	1.11 ± 0.12	1.15 ± 0.13	0.159
ω-6 PUFA / ω-3 PUFA	3.04 ± 0.50	3.06 ± 0.56	3.02 ± 0.48	0.740

Table 4-5 Fatty acid profile of dried blood spots

	All (n=88)	Males (n = 30)	Females (n = 58)	*p*
Omega 3 Polyunsaturated Fatty Acids (ω-3 PUFA)				
18:3ω-3 (ALA, alpha-Linolenic acid)	5.3 ± 1.1	5.3 ± 1.1	5.3 ± 1.1	0.975
20:5ω-3 (EPA, Eicosapentaenoic acid)	0.6 ± 0.7	0.5 ± 0.2	0.6 ± 0.8	0.399
22:5ω-3 (DPA, Docosapentaenoic acid)	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	**0.050**
22:6ω-3 (DHA, Docosahexaenoic acid)	1.3 ± 0.5	1.2 ± 0.5	1.3 ± 0.5	0.643
TOTAL ω-3 PUFA	7.7 ± 1.5	7.6 ± 1.4	7.8 ± 1.6	0.728
Omega 6 Polyunsaturated Fatty Acids (ω-6 PUFA)				
18:2ω-6 (LA, Linoleic acid)	13.8 ± 1.6	13.7 ± 1.6	13.9 ± 1.6	0.641
18:3ω-6 (GLA, γ-Linolenic acid)	1.2 ± 0.4	1.1 ± 0.4	1.2 ± 0.5	0.328
20:2ω-6 (Eicosadienoic acid)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.409
20:3ω-6 (DGLA, Dihomo-γ-linolenic acid)	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.3	0.850
20:4ω-6 (AA, Arachidonic acid)	6.4 ± 1.4	6.5 ± 1.5	6.3 ± 1.4	0.491
22:4ω-6 (Adrenic Acid)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.193
22:2ω-6 (Docosadienoic acid)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.977
TOTAL ω-6 PUFA	22.8 ± 2.8	22.8 ± 2.8	22.8 ± 2.6	0.986
Monounsaturated Fatty Acids (MUFA)				
14:1ω-5 (Myristoleic acid)	14.4 ± 2.1	14.1 ± 2.4	14.5 ± 2.0	0.319
16:1ω-7 (Palmitoleic acid)	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.3	0.427
17:1ω-7 (Cis-10-Heptadecenoic acid)	2.4 ± 0.7	2.3 ± 0.9	2.5 ± 0.6	0.326
22:1ω-17 (5Z-docosenoic acid)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.687
18:1ω-9 (Oleic acid)	2.8 ± 1.0	2.9 ± 1.0	2.8 ± 1.0	0.812
18:1ω-9 (9-octadecenoic acid)	0.7 ± 0.4	0.7 ± 0.4	0.8 ± 0.40	0.559
20:1ω-9 (Gondoic acid)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.663
TOTAL MUFA	22.5 ± 3.2	22.0 ± 3.5	22.7 ± 3.0	0.327
Saturated Fatty Acids (SFA)				
13:0 (Tridecanoic acid)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.447
14:0 (Myristic acid)	3.1 ± 1.2	3.3 ± 1.2	3.1 ± 1.2	0.528
15:0 (Pentadecanoic acid)	0.9 ± 0.3	1.0 ± 0.3	0.9 ± 0.2	0.146
16:0 (Palmitic acid)	22.8 ± 2.1	23.0 ± 2.0	22.7 ± 2.2	0.540
18:0 (Stearic acid)	19.2 ± 1.4	19.4 ± 1.4	19.1 ± 1.4	0.235
20:0 (Arachidic acid)	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.391
22:0 (Behenic acid)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.410
TOTAL SFA	47.0 ± 2.8	47.6 ± 2.7	46.6 ± 2.9	0.188

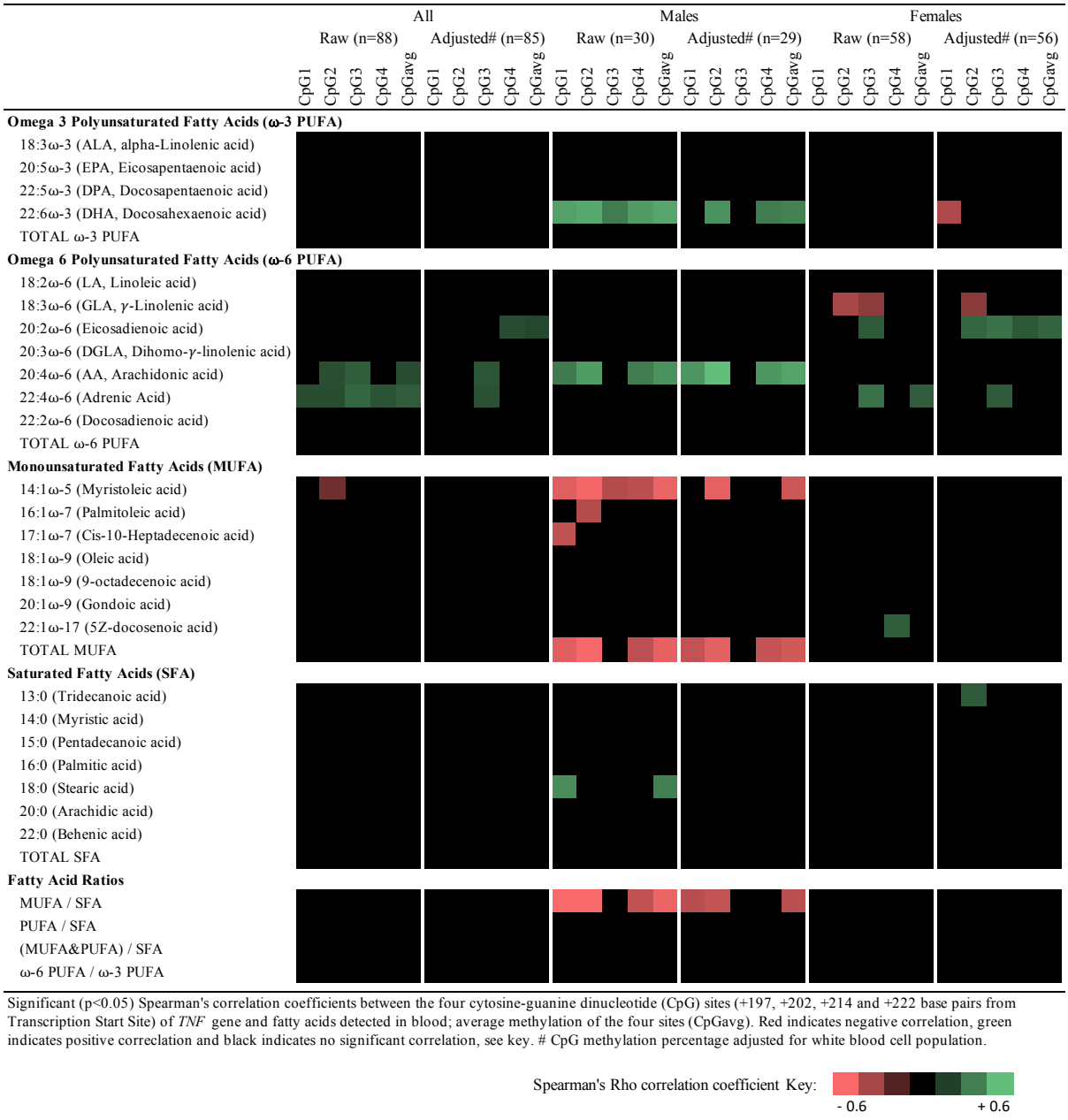
Data is presented as Percentage Total Fatty Acids (%TFA), mean ± standard deviation; differences between groups assessed by independent samples t-test.

4.3.4 Relationship between blood fatty acid and TNF DNA methylation

The relationship between capillary blood fatty acid levels and DNA methylation of *TNF* (correlation) at the four sites studied in exon 1 is highly dependent upon the sex of the cohort. Consequently, examining the results of the cohort as a whole could lead to misleading conclusions and hence the following analysis considers the results according to sex. In addition, it was also found that levels of correlation depended upon whether methylation was adjusted for white blood cell composition (adjustment).

As regards males, the ω-3 PUFA DHA was positively correlated with CpG methylation of *TNF* at all the CpG sites studied. Post adjustment the correlation remained for CpG2 and CpG4 but was no longer observed at CpG1 or CPG3. The ω-6 PUFA arachidonic acid was also found to be positively correlated at all sites except CpG3 where no correlation was observed. Post-adjustment the observed correlations increased. The results for the male cohort also indicated positive correlation at CpG2 and CpG4 for SFA stearic acid. This correlation was no longer evident post adjustment. The male cohort had a negative correlation between the ω-5 MUFA myristoleic acid across all sites pre-adjustment, correlation only remained at CpG2 post-adjustment.

As regards females, no correlations were observed between ω-3 PUFAs and CpG methylation pre-adjustment. Post-adjustment a negative correlation was observed at CpG1. A negative correlation was also observed in respect of the ω-6 PUFA γ-linolenic acid (GLA) at CpG1 and CpG2 pre-adjustment. Post-adjustment the correlation only remained at CpG2. In contrast the ω-6 PUFAs adrenic acid and eicosadienoic acid had a positive correlation. Both adrenic acid and eicosadienoic acid were positively correlated at CpG3 pre-adjustment. Post adjustment these correlations remained but eicosadienoic acid was also found to be positively correlated at CpG2 and CpG4. Additional positive correlations were measured for the MUFA 5Z-docosenoic acid at CpG4 pre-adjustment and for the SFA tridecanoic acid at CpG2 post-adjustment.



Significant ($p<0.05$) Spearman's correlation coefficients between the four cytosine-guanine dinucleotide (CpG) sites (+197, +202, +214 and +222 base pairs from Transcription Start Site) of *TNF* gene and fatty acids detected in blood; average methylation of the four sites (CpGavg). Red indicates negative correlation, green indicates positive correclation and black indicates no significant correlation, see key. # CpG methylation percentage adjusted for white blood cell population.

Figure 4-2 Spearman correlation coefficients heat map between the DNA methylation of four Cytosine-Guanine dinucleotides (CpG) sites located in exon 1 of the Tumor Necrosis Factor (TNF) gene and dried blood spot fatty acid levels measured as a percentage of total fatty acids. Data values shown in Appendix 1.

4.4 Discussion

The research reported here demonstrates that there are significant relationships between levels of specific blood fatty acids and DNA methylation of four CpG sites in exon 1 of the cytokine encoding gene *TNF*. Although methylation levels were not shown to be associated for most of the fatty acids measured, dependent upon the fatty acid group, some positive and some negative associations were observed. Significantly the magnitude and direction of associations were found to differ according to sex.

The CpG sites investigated here have previously been shown to be associated with TNFα mRNA and circulating TNFα (Hermsdorff *et al.*, 2013; Marques-Rocha *et al.*, 2016). The average methylation levels observed in the current study (8.7%) are comparable to those previously observed (Hermsdorff *et al.*, 2013), with a similar profile where the fourth CpG site had slightly higher methylation (12.3%). DNA methylation is associated with the silencing of genes, specifically when the methylation occurs around the transcription start site and the first exon (Brenet *et al.*, 2011), as well as in gene bodies and non-promotor regions (Lou *et al.*, 2014). Multiple sites within the *TNF* gene have been shown to be associated with environmental stimuli and biomarkers for phenotypes (Gazzar *et al.*, 2008; Pieper *et al.*, 2008; Campión *et al.*, 2009; Cordero *et al.*, 2011; Gowers *et al.*, 2011; Nimmo *et al.*, 2012). Additionally, the methylation of these sites in particular have been shown to be potential biomarkers of the adiposity status of an individual (Marques-Rocha *et al.*, 2016). Associations between the methylation status of these sites and TNFα mRNA, as well as cytokine levels, have been observed (Hermsdorff *et al.*, 2013). Although gene expression was not measured in the current study, it can be hypothesise that the fatty acids with a positive relationship to methylation at these sites interact with *TNF* through altered DNA methylation patterns. This may provide an anti-inflammatory effect through reduced TNFα cytokine expression; and vice versa for those with a negative relationship. In this context, and the results presented in this research, methylation of *TNF* may play an important role in inflammation, specifically relating to obesity.

We found that ω-6 PUFA, AA was positively correlated with *TNF* methylation in males, whilst in females it was not correlated, but its elongated form Adrenic Acid was. This

is interesting given that these two fatty acids only differ in length by two carbon atoms. In addition to the positive associations observed with ω-6 PUFAs, in males it was found that there was a positive relationship at all sites between ω-3 PUFA DHA and *TNF* methylation. By contrast, females did not show any significant association with DHA until adjusted for WBC population, when CpG1 showed a significant negative correlation. The differences between the associations observed may be the result of the efficiency in which males and females metabolise fatty acids (Lohner *et al.*, 2013). An alternative explanation could involve the metabolism of fatty acids to eicosanoids, which have also been shown to differ between males and females (Pace, Sautebin and Werz, 2017). It is also important to consider that the sex differences could be the result of differential regulation of *TNF* between males and females; previous research into *TNF* promotor methylation, in a weight loss response study, also demonstrated sex-specific results (Campión *et al.*, 2009).

One of the ways in which fatty acids have been found to modulate inflammation is through altered cytokine expression (Calder, 2003, 2012a; Rangel-Huerta *et al.*, 2012). ω-3 PUFA supplementation and *in vitro* studies report decreased production of TNFα in LPS-stimulated cells when treated with DHA (Weldon *et al.*, 2007). Despite ω-6 PUFAs and their inflammatory mediators being predominantly pro-inflammatory (Calder, 2006), AA-derived eicosanoid 2-series prostaglandins also have a potent anti-inflammatory effect through the inhibition of TNFα production (Miles, Allen and Calder, 2002). As increased methylation of exon 1 is associated with lower levels of TNFα mRNA (Hermsdorff *et al.*, 2013), this research suggests that higher levels of ω-6 PUFAs AA and Adrenic Acid as well as the ω-3 PUFA DHA result in higher methylation of *TNF*, and this could explain a mechanism through which these fatty acids interact with inflammation. Further investigation of the associations found here may lead to a better understanding of this interaction and its mechanism.

Work carried out by Hermsdorff *et al.* on the same CpG sites found an association with ω-6 PUFA intake, however their research found the direction of the association to be the opposite to that observed here (Hermsdorff *et al.*, 2013). It is possible that this discrepancy arises from the different methodologies used for the measurement of the ω-6 PUFAs. Hermsdorff *et al* measured ω-6 dietary intake, rather than the biochemical

measurement of blood fatty acids used here. Multiple factors impact upon the fats within blood including the proportion of other dietary fats and other dietary components (Katan, Zock and Mensink, 1994), endogenous synthesis of fatty acids and the individual's genetic makeup (Porenta *et al.*, 2013). These factors and the inherent disadvantages of FFQ (Prentice *et al.*, 2011) compared to biochemical measurements may explain the differences in results.

In comparison to the ω-6 PUFA relationship that was observed, the MUFA myristoleic acid was found to have a weak negative correlation with *TNF* methylation at CpG2. Males had a significant negative association between *TNF* methylation and myristoleic acid, as well as total MUFA; no relationship was observed in the female cohort. MUFAs are generally considered the healthier option to SFAs (Schwingshackl and Hoffmann, 2014), however negative association was observed between MUFA/SFA ratio and DNA methylation. No other fatty acid ratios showed significant relationships. The differing effects of ω-6 PUFAs and MUFAs on the methylome have been shown in *in vitro* monocyte models. AA had a trend towards global hypermethylation and the MUFA Oleic acid (OA) had a (weaker) hypomethylating effect on the methylome. The changes observed were in biologically significant sites rather than random sporadic effects throughout the genome (Silva-Martínez *et al.*, 2016).

Consideration must be given to the design of this research. Firstly, the biological significance of measuring only four CpG sites must be considered. The four CpG sites are located close to NFKB transcription binding sites and therefore alterations in their methylation may impact gene transcription. Secondly, it is unclear if the absolute percentage differences observed in this data set are substantial enough to be biologically significant, and this warrants further investigation. Although previous research has demonstrated associations with *TNF* transcripts, by not measuring the levels in this research there is a limitation to the conclusions that can be drawn. In addition, measurement of other CpG sites within the *TNF* gene would provide further detail into the epigenetic control of this cytokine's expression. However, due to the structure of the *TNF* gene there are complications in designing an assay to measure methylation within the promotor of *TNF*. The *TNF* promotor has multiple homologous stretches of sequence when the DNA is bisulphite-converted, there are few methods which allow for sequencing when this is the case.

There are methodological considerations for DNA sources used within epigenetic research due to the specificity of the epigenetic makeup of each individual cell. For example, there is a greater difference in methylation between tissues from the same individual than there are from the same tissue from multiple individuals (Byun *et al.*, 2009). In the current research blood was used as the DNA source, which is a tissue heterogenous in cell composition. Nevertheless, there are benefits in using blood as a source of DNA, firstly it is convenient to collect through a fingerprick sample, and secondly it contains WBCs which play key roles within the inflammatory process. To combat the heterogeneity of the cells and to minimise WBC composition as a confounding factor, the methylation results have been presented both as raw and as adjusted for WBC composition using a regression-based method (Jones *et al.*, 2015). The relationships between the fatty acids and *TNF* methylation did not disappear once adjusted for WBC composition.

Although individuals were not excluded based on their health status, participants were required to travel to the National Centre for Sport and Exercise Medicine in Loughborough for data and sample collection and consequently the cohort was generally healthy. Investigating the associations between *TNF* methylation and fatty acids in an unhealthy cohort could further elucidate the epigenetic effect of fatty acids with inflammation. Unlike the wide range of PUFA status that the Yup'ik study utilised, the cohort in this chapter displayed a small range in ω-3 PUFAs. Additionally, the ω-6/ω-3 PUFA ratio was low in comparison to that expected for a Western population (Simopoulos, 2016). Future research should investigate whether interventions could alter the methylation profile of the *TNF* gene to one that is associated with a healthier phenotype. For example, could altering the fatty acid composition of blood through dietary supplementation increase the methylation percentage of *TNF* to give a protective effect against prolonged inflammation, a characteristic of obesity and chronic disease? The methylation of *TNF* has previously been shown to decrease with age (Gowers *et al.*, 2011), but this was not possible to measure in this cohort, which although having a wide age range, was skewed towards younger individuals.

The increasing prevalence of non-communicable diseases places increased pressure on society, healthcare providers and governments worldwide (Naghavi *et al.*, 2017). A common component of these diseases is chronic inflammation. It is of great

importance to consider whether health outcomes can be improved in order to alleviate some of this burden. The potential to make small changes to diet which could have a positive impact upon the reduction of chronic incapacitating diseases, including through interactions with the inflammatory process, is attractive, and a better understanding of epigenetic effects may be of assistance. In conclusion, this research indicates that the levels of certain blood fatty acids are associated with the epigenetic signatures, DNA methylation, of *TNF* exon 1.Further research into how fatty acids interact with the epigenetic control of cytokines may provide a therapeutic avenue for prevention of chronic inflammation. Additionally, the results here suggest that subsequent research must include the analysis of results separately between the sexes in order to not miss important findings that may be sex-specific.

Chapter 5 Does ω-3 PUFA supplementation alter the methylation status of genes encoding inflammatory cytokines TNFα, IL6 and IL1β?

5.1 Introduction

The cross-sectional analysis reported in Chapter 4 demonstrated that there are associations between the amounts of fatty acids in blood and the degree to which four CpG sites within *TNF* are methylated. Increased methylation of these CpG sites has been associated with reduced *TNF* gene expression (Hermsdorff *et al.*, 2013), and since this encodes for a cytokine which is pro-inflammatory, would be expected to reduce inflammation. Chronic inflammation is a concomitant of many debilitating chronic diseases including, for example, cancer, CVD, chronic obstructive pulmonary disease (COPD) and type 2 diabetes (Hotamisligil, 2006; Shoelson, 2006; van der Molen, 2010; Rook and Dalgleish, 2011). Currently nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to control inflammation but it has become increasingly evident that they may have unintended adverse effects. As a consequence, there is now increasing interest in controlling symptoms of chronic diseases, including inflammation, in a 'more natural' way.

Against this background, and in the light of the associations reported in Chapter 4, it is relevant to ask whether supplementing the human diet with fatty acids could increase their levels in the blood and therefore influence the methylation level of the four CpG sites that were investigated. If this was the case, then it might be possible to reduce chronic inflammation, at least to some extent, by making relatively simple changes to diet. A better understanding of the epigenetic mechanism of the genome and the way in which environmental factors, including diet, control gene expression could provide a route to reducing dependency upon NSAIDs and alleviate the unintended consequences of their use.

Research into the effect of ω-3 PUFA supplementation has thus far investigated epigenome-wide methylation via 450k arrays (Amarasekera *et al.*, 2014; Aslibekyan *et al.*, 2014; Lind *et al.*, 2015; Voisin *et al.*, 2015; van Dijk *et al.*, 2016; Arpón *et al.*, 2017; Perfilyev *et al.*, 2017; Tremblay *et al.*, 2017; McMorrow *et al.*, 2018) or targeted specific genes, for example those encoding fatty acid metabolism enzymes, e.g. *FADS1* and *FADS2* (do Amaral *et al.*, 2014; Hoile *et al.*, 2014; Cui *et al.*, 2016; Rahbar *et al.*, 2018). The 450k array measures the methylation level of more than 450,000 single CpG sites dispersed throughout the genome. Analysis of 450k array data is prone to multiple testing errors, as well as possible cross-reactivity and polymorphisms in CpG sites (Chen *et al.*, 2013). However, they are useful in identifying DMRs that may have important biological functions, and their results can be used to identify enriched pathways and future targets for investigation. ω-3 PUFA supplementation studies that have utilised the 450k arrays have identified multiple DMRs as a result of the intervention (Arpón *et al.*, 2017; Perfilyev *et al.*, 2017; Tremblay *et al.*, 2017; McMorrow *et al.*, 2018). A large number of pathways where DMRs were found are altered by supplementation and these include those that are related to the inflammatory and immune response, lipid metabolism, cardiovascular signalling and those implicated in type 2 diabetes, amongst others.

Currently, to the authors knowledge, the following genes have been the only targets of methylation analysis in adult supplementation studies: *FADS1, FADS2, ELOVL2, ELOVL5, CD14, PDK4, CD36,* and *FFAR3 "Free Fatty Acid Receptor 3"*. The methylation of *FADS1* promotor has been shown to increase as a result of supplementation of ω-3 PUFAs compared to a control group in a calorie-restricted intervention (do Amaral *et al.*, 2014). However, in separate research by Hoile *et al.* (2014), where supplementation was the only intervention and the intervention was carried out in two separate cohorts, no significant changes were observed in FADS1. These two studies targeted different CpG sites within the *FADS1* gene and this may be responsible for the different results obtained. The response of *FADS2* methylation to either olive oil or ω-3 PUFA supplementation was found to be sex-specific. Increases in *FADS2* methylation were observed in females post-supplementation at four CpG sites, as opposed to males where one site increased and one decreased. Hoile *et al.*, (2014) found that olive oil supplementation increased the methylation at more CpG sites than ω-3 PUFA supplementation. The same result was observed for

ELOVL5 (at fewer CpG sites), and notably the methylation of *FADS2* and *ELOVL5* CpG sites was negatively associated with their mRNA transcripts.

Supplementation studies that have investigated the effect of ω-3 PUFAs on pro-inflammatory cytokines have reported that the production of cytokines TNFα, IL1β and IL6 is reduced post-supplementation (Li *et al.*, 2014; Tan *et al.*, 2018). However, as discussed in Chapter 1, the full mechanism through which this is achieved is not currently understood. In this chapter, the aim was to investigate the effect of ω-3 PUFA supplementation on epigenetic signatures *in vivo* of the inflammatory cytokine genes *TNF*, *IL6* and *IL1B*.

5.2 Methods

5.2.1 Ethical approval and study requirements

Due to differences in cell membrane incorporation of ω-3 PUFAs between the sexes and because of difficulties fitting the testing schedule within the menstrual cycle, which could alter the blood markers being measured, it was decided that the cohort for this study would be males.

Prior to the recruitment of participants ethical clearance for the study was sought from, and granted by, the Loughborough University Ethics Approvals (Human Participants) Sub-Committee (reference R16-P138). Recruitment was then achieved through word of mouth, posters, emails and social media advertisements in the Loughborough area. Potential participants attended the National Centre for Sport and Exercise Medicine prior to starting the study. During this preliminary session they were fully informed of the study requirements and assessed for study suitability prior to providing written consent. Participants then completed a health screening questionnaire, ethnicity questionnaire, FFQ and the International Physical Activity Questionnaire (IPAQ).

In order to be included in the study the participants had to meet the following criteria:

- Male (see above)
- Aged between and including 18 to 30 years.
- Body mass index (BMI; in kg/ m2), between and including 18 to 30.
- No consumption of vitamin or ω-3 PUFAs supplements in the previous 6 months.
- A low habitual oily fish consumption (less than 4 servings per month), however this was relaxed when recruitment proved difficult.
- Non-smoking.
- No plan to change dietary habits.
- No significant changes in body weight in the past 6 months.
- No habitual use of anti-inflammatory drugs.
- No history of diabetes, heart disease, coagulation/bleeding disorders, metabolic disease or serious allergy.

- Not known to have blood-borne virus.
- No receipt of inoculations within 2 months of starting the study or an intention to receive any during the duration of the intervention.
- No donation of or intention to donate blood within 8 weeks of providing the first study sample or at any point during the intervention period.
- Not allergic to fish.

5.2.1.1 *Participants*

A total of 39 individuals indicated their interest in the study. Of these 12 did not respond to follow up emails and eight did not fit the inclusion criteria. Of the remaining 19 who commenced participation, one was unable to complete as he displayed needle phobia and another contracted an illness, which although unrelated to the study, prevented them from continued participation. A total of 17 males (aged 22.56 ± 2.76 yrs; body mass 80.07 ± 9.02 kg, mean ± standard deviation) completed the study.

5.2.2 Study design

The study involved five laboratory visits over the course of 28 days. The first visit was used to take baseline measurements and samples, the participants then undertook a course of ω-3 PUFA supplementation for 28 days, visiting the laboratory on days 7, 14, 21 and 28, as shown in Figure 5-1. Throughout the course of the study, participants were asked to maintain their habitual diet (including where applicable their usual consumption of fatty fish), weight and activity levels.

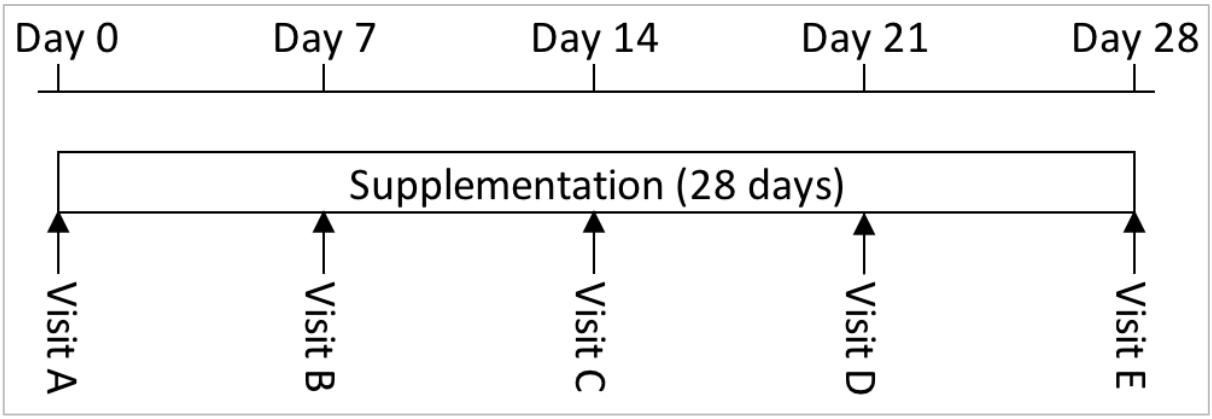


Figure 5-1 Intervention outline for the 28-day supplementation period and five laboratory visits.

5.2.3 Supplementation

For 28 days, participants took four ω-3 PUFA capsules (Omega Via, Innovix Pharma Inc, USA) which contained a total of 6000mg of fat, including 4420mg total ω-3. The ω-3 component comprised 3,120mg EPA, 1,040mg DHA, and 260mg of other ω-3 PUFAs, Table 5-1. It was recommended that participants took two capsules in the morning and two in the evening, each with a meal.

Table 5-1 Fatty acid composition of the omega-3 polyunsaturated fatty acids (ω-3 PUFAs) supplements (Omega Via, Innovix Pharma Inc, CA, USA)

Supplement contents	Per capsule (mg)	Per four capsules (mg)
Omega 3	1105	4420
EPA	780	3120
DHA	260	1040
DPA and other ω-3 PUFAs	65	260
Total capsule (mg)	1500	6000

Eicosapentaenoic acid (EPA); Docosapentaenoic acid (DPA); Docosahexaenoic acid (DHA).

5.2.4 Study protocol

A food diary was completed by each participant for the day prior to the first laboratory visit, this food diary was then used to replicate the same dietary intake the day before each of the subsequent laboratory visits. On laboratory visit days, participants were asked to take their supplements after they had visited the laboratory that day. Participants were also asked to abstain from the following before attending the laboratory:

- Consumption of food and drink (exc. water) for 12 hours
- vigorous exercise for 24 hours
- alcohol for 48 hours

Laboratory visits lasted 30 minutes and were conducted between 7am and 10am.

5.2.4.1 *Anthropometric measurements*

Each visit commenced with a recording of anthropometric measurements. Height was measured using a digital stadiometer to the nearest 0.1 centimetre (cm) and weight using digital weighing scales to the nearest 0.01 kilogram (kg). Bioelectrical

impedance analysis (BIA) was used to estimate body composition (fat, water and muscle). Using a measuring tape, waist and hip circumference was measured to the nearest 0.1 centimetre (cm) and used to calculate waist-to-hip ratio.

5.2.4.2 *Blood pressure*

Using a digital automatic blood pressure monitor the arterial blood pressure was measured by placing the cuff on the upper right arm with the arm resting on a pillow next to the participant. Participants remained in a Fowler's position for five minutes prior to the first measurement. Three measurements of blood pressure were taken with an interval of one minute between each measurement. An average of the three measurements was used for analysis.

5.2.4.3 *Blood collection and handling*

Participants remained in the Fowler's position for venous blood samples. Samples were collected from the antecubital vein via venepuncture at the end of each laboratory visit. A total of 70ml of blood was collected into EDTA coated vacutainers (Becton, Dickinson & Company, NJ, USA). The vacutainers were gently inverted eight to 10 times. Aliquots of blood were taken from one of the vacutainers for a cell count (Beckman Coulter Counter, USA); in addition, aliquots of blood were frozen at -80oC for DNA extraction (section 5.2.7). Six vacutainers were processed to isolate PBMCs, see section 5.2.5 for details.

5.2.5 PBMC isolation

PBMCs were isolated for fatty acid composition analysis and *in vitro* stimulation method development (not completed within this thesis, details of the isolation, cyroprservation given in Appendix 3). A blood sample was collected at each visit into 10ml EDTA vacutainers. PBMCs were extracted using density gradient separation and resuspended. For fatty acid analysis the cells were resuspended in 250μL PBS per 10ml blood tube. Cells were counted using a cell counter (Beckman Coulter Counter, USA) prior to being stored at -80^{o}C until required for further analysis.

5.2.6 Fatty acid measurements

The fatty acid composition of PBMCs was measured from samples collected at baseline (day zero) and each subsequent lab visit. Fatty acid incorporation was analysed by GC-MS to profile the fatty acid composition of the participants' PBMCs. Briefly, the PBMCs were homogenised and 100μL of the resulting suspension was derivatised as described in section 4.2.3. Fatty acid quantities were determined based on the relative abundance to the internal standard. Fatty acids levels were calculated as %TFA.

5.2.7 Sample preparation and DNA extraction

Prior to the blood samples being frozen, a blood cell count was obtained from each sample. Whole blood was used for genomic DNA extraction and subjected to bisulphite conversion using EpiTect® Fast LyseAll Bisulphite conversion kit (Qiagen, Germany) to the manufacture's protocol. Briefly, blood samples were lysed and proteins denatured, the resulting pellets were re-suspended in PBS and added directly into the bisulphite reaction. The DNA was sodium bisulphite-treated and subjected to two cycles of denaturing at 95°C for 5 minutes and incubation 60°C for 20 minutes. Bisulphite-converted DNA was desulfonated and purified using MinElute spin columns.

5.2.8 DNA methylation analysis

DNA was extracted from blood and bisulphite-converted using EpiTect LyseAll kit (Qiagen, Germany). DNA methylation levels of *TNF*, *IL1B* and *IL6* were measured by pyrosequencing using the PyroMark Q48 Autoprep (Qiagen, Germany) with the assays designed and validated in Chapter 3. Data is presented as mean ± standard deviation.

5.2.9 Statistics

The one-way repeated ANOVA is sensitive to departures from sphericity, an assumption that it is not easily detected in small sample sizes (Maxwell and Delaney, 2003); consequently, here the Greenhouse-Geisser correction was applied to all ANOVA p values reported.

Analysis was completed both with and without extreme outliers (3 box lengths from the edge of the box plot) in the data; where no impact of the outliers on significance was observed, then the results, including the outliers, is presented.

Two participants missed one lab visit each, in order for their data to be included in the one-way repeated ANOVA, an average was taken from the data point of each visit either side of the missed lab visit. The same method was used for replacing outliers

5.3 Results

5.3.1 Sample demographic

A total of 17 participants completed the four weeks of supplementation. There were no significant differences in participant characteristics between baseline and post-supplementation at week four, Table 5-2. No change in diastolic blood pressure over the 28 days supplementation, Table 5-3, however there was a small non-significant decrease in systolic blood pressure over the intervention (130 ± 10 to 125. ± 9). Additionally, there was a significant decrease in heart rate from day 7 (60 ± 11) and day 14 (59 ± 10) compared to day 28 (55 ± 10).

Table 5-2 Sample demographic pre and post 28-day ω-3 PUFA supplementation

	Before ω-3 PUFA (n = 17)	After ω-3 PUFA (n = 17)	*p*
Weight (kg)	80.1 ± 9.0	80.6 ± 9.3	0.148
BMI (kg/m^2)	24.0 ± 2.3	24.2 ± 2.4	0.170
Waist circumference (cm)	79.5 ± 5.2	79.6 ± 5.1	0.927
Hip circumference (cm)	87.5 ± 6.0	87.4 ± 5.4	0.910
Waist-to-Hip ratio	0.9 ± 0.1	0.9 ± 0.1	0.952
Skeletal Muscle Mass (kg)	32.9 ± 2.8	33.0 ± 2.6	0.893
Water weight (L)	47.7 ± 3.8	48.9 ± 4.5	0.122
Body fat (kg)	17.0 ± 4.3	17.1 ± 4.5	0.687

Data presented before and after 28-day omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation
Mean ± standard deviation; differences between groups assessed by independent samples t-test.

Table 5-3 Blood pressure and heart rate during a four-week supplementation with omega-3 polyunsaturated fatty acid

Variable	Day 0	Day 7	Day 14	Day 21	Day 28	*p*
Diastolic blood pressure (mmHG)	69 ± 6	71 ± 5	68 ± 7	69 ± 8	67 ± 5	0.237
Systolic blood pressure (mmHG)	130 ± 10	130 ± 7	129 ± 8	127 ± 9	125 ± 9	0.072
Heart Rate (beats per minute)	57 ± 11 **a,b**	60 ± 11 **b**	59 ± 10 **b**	58 ± 11 **a,b**	55 ± 10 **a**	**0.022**

n = 16; Data is presented as mean ± standard deviation. Significant difference assessed by ANOVA with Greenhouse-Geisser correction applied. Means that do not share a letter (a,b) are significantly different ($p < 0.05$) from each other after correcting for multiple testing (Bonferroni correction).

5.3.2 Fatty acid profile of peripheral blood mononuclear cells (PBMCs)

The fatty acid profile of PBMCs was measured at baseline and every seven days of the intervention; results are presented in Table 5-4. ω-3 PUFA, EPA significantly increased during the first seven days of the supplementation from 0.1 ± 0.1 to 0.5 ± 0.3 %TFA and continued to increase (not with statistical significance) until day 28 (0.7 ± 0.3 %TFA). In addition to the EPA increase, DPA significantly increased from day 0 (0.4 ± 0.2 %TFA) compared to day 7 (0.7 ± 0.2 %TFA) and DHA increased over the 28 days from 0.5 ± 0.2 to 0.6 ± 0.2 %TFA, not with statistical significance. The result of the EPA, DPA and DHA increases is a significant increase in total ω-3 PUFA within PBMCs by day 21 (9.4 ± 0.7 %TFA) compared to baseline (8.1 ± 1.1 %TFA). There was no change in the levels of the essential fatty acid alpha-linolenic (ALA) over the 28 days of supplementation.

During the first 14 days of supplementation there was a significant decrease in the levels of ω-6 PUFA adrenic acid from 0.4 ± 0.2 to 0.2 ± 0.1 %TFA. There was a significant change in dihomo-γ-linolenic acid (DGLA), which did not remain significant after correction for multiple testing.

5.3.3 CpG methylation of TNF, IL6 and IL1B

Methylation of *TNF*, *IL6* and *IL1B* was measured every seven days throughout the intervention. No change was observed in any of the three genes across the four weeks supplementation.

Table 5-4 Peripheral blood mononuclear cell fatty acid levels before ω-3 supplementation and every 7 days for 28 days,

	Day 0	Day 7	Day 14	Day 21	Day 28	*p*
Omega 3 Polyunsaturated Fatty Acids (ω-3 PUFA)						
18:3ω-3 (ALA, alpha-Linolenic acid)	7.0 ± 0.8	7.3 ± 0.7	7.2 ± 0.7	7.3 ± 0.6	7.0 ± 0.6	0.495
20:5ω-3 (EPA, Eicosapentaenoic acid)	0.1 ± 0.1 **a**	0.5 ± 0.3 **b**	0.6 ± 0.3 **b**	0.7 ± 0.2 **b**	0.7 ± 0.3 **b**	**<0.001**
22:5ω-3 (DPA, Docosapentaenoic acid)	0.4 ± 0.2 **a**	0.7 ± 0.2 **b**	0.7 ± 0.3 **a,b**	0.8 ± 0.2 **b**	0.8 ± 0.2 **b**	**0.001**
22:6ω-3 (DHA, Docosahexaenoic acid)	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.3	0.072
TOTAL ω-3 PUFA	8.1 ± 1.1 **a**	9.1 ± 0.9 **a,b**	9.0 ± 1.4 **a,b**	9.4 ± 0.7 **b**	9.1 ± 1.0 **a,b**	**0.007**
Omega 6 Polyunsaturated Fatty Acids (ω-6 PUFA)						
18:2ω-6 (LA, Linoleic acid)	2.6 ± 0.9	2.5 ± 0.5	2.5 ± 1.0	2.9 ± 0.7	2.7 ± 0.7	0.490
18:3ω-6 (GLA, γ-Linolenic acid)	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.3	0.794
20:2ω-6 (Eicosadienoic acid)	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.160
20:3ω-6 (DGLA, Dihomo-γ-linolenic acid)	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	**0.047**
20:4ω-6 (AA, Arachidonic acid)	5.8 ± 2.1	5.6 ± 2.0	5.2 ± 2.0	5.9 ± 1.3	5.2 ± 1.3	0.660
22:4ω-6 (Adrenic Acid)	0.4 ± 0.2 **a**	0.3 ± 0.2 **a,b**	0.2 ± 0.1 **b**	0.2 ± 0.1 **b**	0.2 ± 0.1 **b**	**0.001**
22:2ω-6 (Docosadienoic acid)	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.806
TOTAL ω-6 PUFA	10.5 ± 3.0	9.9 ± 2.3	9.5 ± 2.9	10.5 ± 1.7	9.7 ± 1.8	0.589
Monounsaturated Fatty Acids (MUFA)						
14:1ω-5 (Myristoleic acid)	13.4 ± 5.1	14.1 ± 4.2	14.5 ± 4.4	12.7 ± 3.2	13.3 ± 2.8	0.662
16:1ω-7 (Palmitoleic acid)	3.4 ± 3.2	3.4 ± 3.0	2.6 ± 0.9	3.1 ± 3.2	2.5 ± 0.5	0.371
17:1ω-7 (Cis-10-Heptadecenoic acid)	2.2 ± 0.9	2.5 ± 1.1	2.4 ± 0.9	2.0 ± 0.6	2.1 ± 0.6	0.285
22:1ω-17 (5Z-docosenoic acid)	0.3 ± 0.2	0.4 ± 0.4	0.3 ± 0.2	0.5 ± 0.8	0.4 ± 0.5	0.493
18:1ω-9 (Oleic acid)	8.3 ± 3.2	8.5 ± 3.2	8.9 ± 2.9	7.7 ± 2.7	8.0 ± 2.4	0.669
18:1ω-9 (9-octadecenoic acid)	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.510
TOTAL MUFA	28.1 ± 10.0	29.4 ± 8.7	29.2 ± 8.7	26.5 ± 7.6	26.7 ± 6.1	0.661
Saturated Fatty Acids (SFA)						
13:0 (Tridecanoic acid)	1.4 ± 0.3	1.4 ± 0.5	1.6 ± 0.8	1.3 ± 0.4	1.4 ± 0.4	0.362
14:0 (Myristic acid)	3.8 ± 1.9	3.3 ± 1.5	3.4 ± 1.3	2.9 ± 0.7	3.2 ± 0.8	0.296
15:0 (Pentadecanoic acid)	2.5 ± 1.2	2.3 ± 1.0	2.2 ± 0.9	1.9 ± 0.5	2.1 ± 0.5	0.333
16:0 (Palmitic acid)	25.8 ± 4.9	24.8 ± 3.8	25.7 ± 3.3	26.1 ± 3.4	26.6 ± 3.0	0.467
18:0 (Stearic acid)	19.2 ± 3.7	19.1 ± 3.5	18.8 ± 4.6	20.6 ± 3.2	20.4 ± 2.7	0.433
20:0 (Arachidic acid)	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.574
21:0 (Heneicosylic acid)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.095
22:0 (Behenic acid)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.718
TOTAL SFA	53.4 ± 8.8	51.6 ± 7.2	52.5 ± 5.9	53.6 ± 6.3	54.5 ± 5.5	0.571

n = 17; Data is presented as Percentage Total Fatty Acids (%TFA), mean ± standard deviation. Significant difference assessed by ANOVA with Greenhouse-Geisser correction applied. Means that do not share a letter (a,b) are significantly different from each other after correcting for multiple testing (Bonferroni correction).

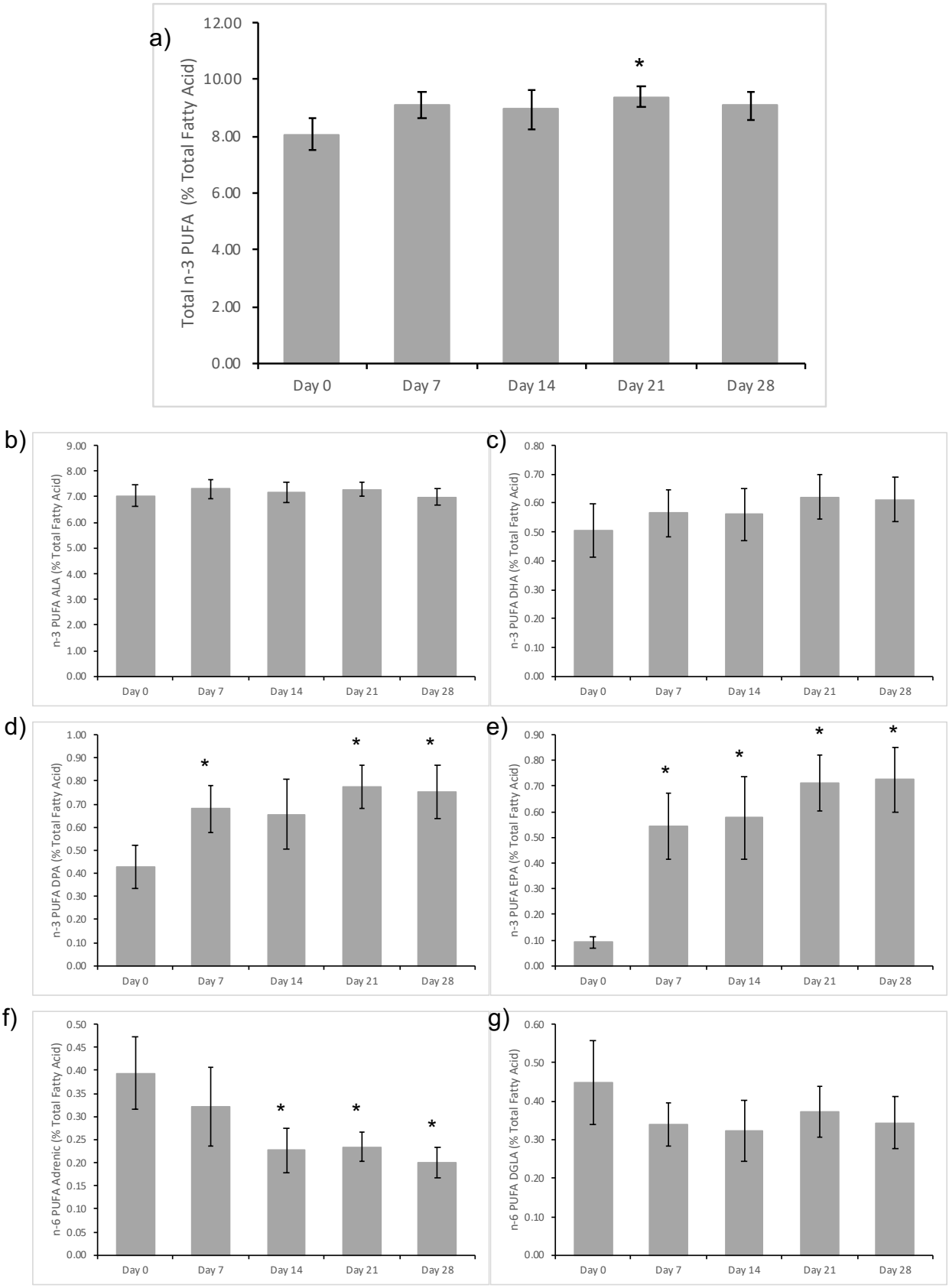


*Figure 5-2 Fatty acid composition of whole blood, n=17. (a) Total omega 3 (ω-3) poly unsaturated fatty acid (PUFA) (b) ω-3 PUFA alpha-Linolenic acid, ALA (c) ω-3 PUFA Docosahexaenoic acid, DHA Data presented as mean ± 95% confidence intervals. * significantly different to day 0.*

Table 5-5 Percentage DNA methylation during the course of a four-week Omega-3 supplementation

	Day 0	Day 7	Day 14	Day 21	Day 28	*p*
Tumor Necrosis Factor (*TNF*, %, n = 17)						
CpG1	9.4 ± 2.7	9.1 ± 3.8	8.6 ± 2.5	8.8 ± 1.9	9.1 ± 2.8	0.770
CpG2	8.0 ± 3.0	7.0 ± 3.3	6.9 ± 2.1	6.9 ± 2.1	7.5 ± 1.8	0.499
CpG3	8.3 ± 3.2	7.8 ± 2.6	7.3 ± 2.1	7.4 ± 1.7	7.9 ± 2.0	0.485
CpG4	14.4 ± 4.5	13.1 ± 4.6	13.0 ± 3.3	13.4 ± 2.9	14.8 ± 4.2	0.395
CpG Average	10.0 ± 3.3	9.3 ± 3.5	8.9 ± 2.4	9.1 ± 1.9	9.8 ± 2.5	0.519
Interleukin-6 (*IL6*, %, n = 11)						
CpG1	91.2 ± 2.3	90.4 ± 2.9	91.1 ± 2.8	90.0 ± 2.9	90.4 ± 2.9	0.460
CpG2	90.8 ± 2.1	91.0 ± 1.4	90.9 ± 1.9	90.6 ± 2.0	91.3 ± 1.4	0.678
CpG3	91.6 ± 1.8	91.7 ± 1.3	91.1 ± 1.9	92.0 ± 1.9	92.6 ± 1.5	0.248
CpG4	87.7 ± 2.0	88.0 ± 1.8	87.7 ± 1.9	87.8 ± 2.0	88.9 ± 1.7	0.330
CpG5	82.8 ± 3.4	83.1 ± 4.0	83.1 ± 2.9	83.1 ± 3.5	82.9 ± 4.1	0.945
CpG6	88.8 ± 3.0	89.2 ± 2.5	89.4 ± 2.5	89.2 ± 2.0	89.7 ± 2.9	0.693
CpG Average	88.8 ± 1.8	88.9 ± 1.7	88.9 ± 1.5	88.8 ± 1.9	89.3 ± 1.6	0.659
Interleukin-1 beta (*IL1B*, %, n = 17)						
CpG1	92.7 ± 3.4	92.9 ± 3.5	92.7 ± 3.5	91.9 ± 4.5	92.4 ± 4.3	0.460
CpG2	94.0 ± 1.4	94.4 ± 1.2	94.5 ± 1.7	93.9 ± 2.6	93.9 ± 3.2	0.678
CpG3	85.8 ± 4.5	87.2 ± 4.1	86.1 ± 4.5	85.9 ± 4.9	85.2 ± 7.2	0.248
CpG4	90.8 ± 4.5	91.2 ± 4.2	90.4 ± 4.5	90.9 ± 3.2	89.7 ± 5.2	0.330
CpG5	75.1 ± 2.5	75.6 ± 2.3	75.8 ± 2.5	76.1 ± 3.1	75.2 ± 3.9	0.945
CpG Average	87.7 ± 2.9	88.3 ± 2.7	87.9 ± 2.9	87.7 ± 2.7	87.3 ± 4.4	0.693

Data is presented as Percentage methylation, mean ± standard deviation; Significant difference assessed by ANOVA and means that do not share a letter are significantly different after correcting for multiple testing (Bonferroni correction)

5.4 Discussion

In a group of young healthy males, this research sought to investigate whether altering dietary fatty acid consumption would have an impact upon the promotor methylation levels of cytokine genes. Dietary supplementation with a high dose of ω-3 PUFAs, EPA and DHA, for a four-week period resulted in significant changes to the levels of certain fatty acids incorporated into PBMCs. However, there were no observed changes to the methylation levels of *TNF*, *IL6* or *IL1B* over that intervention period.

ω-3 PUFAs are known to decrease inflammatory cytokine expression and production through multiple mechanisms as discussed previously in this thesis. The three cytokines investigated here are partly controlled through epigenetic mechanisms. Regulation of *IL6* expression appears to be predominantly controlled through histone modifications, with HDAC and HAT inhibitors reducing or preventing transcription respectively (Hu *et al.*, 2017). However, methylation of CpG sites within the promotor region of *IL6* are associated with gene transcription (Kirchner *et al.*, 2014; Poplutz *et al.*, 2014; Ma *et al.*, 2016).

Elsewhere research undertaken into the effect of ω-3 PUFA supplementation on the methylation profile of genes has also utilised 450k arrays. These studies observed altered methylation in genes linked to inflammatory pathways when baseline levels were compared to those post supplementation (Arpón *et al.*, 2017; Perfilyev *et al.*, 2017; Tremblay *et al.*, 2017; McMorrow *et al.*, 2018). Specifically methylation changes in *IL6* and *TNF* were measured and an increase in methylation was observed as a result of supplementation (Perfilyev *et al.*, 2017). It was not possible to confirm these findings using the targeted approach reported in this chapter. It is difficult to make comparisons between the work reported here and the previous studies carried out elsewhere, or indeed to make comparisons between those studies because of differences in the interventions. This is an issue that has plagued the field of fatty acid research and appears to be responsible for the observed inconsistencies in findings. The four studies referred to above illustrate this problem since they comprised a six week supplementation with a high dose ω-3 PUFA (Tremblay *et al.*, 2017), a seven week RCT overfeeding with PUFA or SFA included (Perfilyev *et al.*, 2017), an eight

week “anti-inflammatory nutrition” intervention which included ω-3 PUFAs (McMorrow *et al.*, 2018), and a 5 year Mediterranean diet intervention (Arpón *et al.*, 2017).

The research reported in Chapter 4 found that methylation of the *TNF* gene promotor was associated with levels of particular fatty acids within whole blood. Specifically, in males the ω-3 PUFA DHA and the ω-6 PUFA AA were associated with methylation levels of *TNF*. In this chapter this research aimed to investigat whether altering the dietary fatty acid intake of a male cohort affects the fatty acid composition of PBMCs and whether this would result in alterations to the methylation signature of the *TNF* gene. Furthermore, it was also investigated whether the increased dietary fatty acid intake impacted upon the methylation levels of two other inflammatory cytokine genes, *IL6* and *IL1B.*

Significantly, the intervention undertaken in this chapter did not give rise within PBMCs to a change in levels of any of the fatty acids, which were shown in chapter 4 as being associated with *TNF* methylation. Had the intervention caused a change in the levels of DHA or AA within PBMCs, then it would have been expected to see corresponding changes to the methylation of *TNF,* however this was not the case and so the fact that the intervention did not lead to a change in methylation of *TNF* is less surprising.

The intervention described in this chapter was intended to give a large dose of ω-3 PUFAs that was expected to change the fatty acid composition of the blood. The fatty acid levels within PBMCs were measured because these cells play a significant role in the immune system and provide a source of DNA for epigenetic analysis. By altering the PBMC fatty acid composition, it was intended to alter the environment of these cells, and therefore their epigenetic regulation on the specific genes studied.

Measuring the fatty acid levels of WBC, compared to whole blood or red blood cells (RBCs) targets the biologically relevant cell type to inflammation. In addition, the incorporation of ω-3 PUFAs into RBC and WBC following ω-3 PUFA supplementation occurs at different rates. RBC incorporation occurs in a linear dose-response relationship, however the incorporation into WBCs is not linear with lower incorporation levels (Witte *et al.*, 2010).

Incorporation of EPA in PBMCs occurs in a dose-dependent manner (Rees *et al.*, 2006), therefore the dose used of 4.42g total ω-3 PUFA (3.12g EPA, 1.04g DHA and

0.26g other ω-3 PUFA) per day, and to the authors knowledge, higher than any previous research that has investigated DNA methylation changes as the result of supplementation (as reported in Table 2-2). This dose was achieved using high strength capsules that only required the participant to take four capsules per day. The incorporation of EPA and DHA into PBMCs reaches near saturation of the PBMCs within the first seven days of supplementation (Faber *et al.*, 2011), with further supplementation only having a modest effect of the composition (Yaqoob *et al.*, 2000). In the current study, EPA and DPA increased significantly from baseline (day 0) to day seven, and although continued to increase until day 28, the further increases were not statistically significant from the previous measurement; consistent with the studies above. The increases observed in DHA levels was small and not statistically significant, in agreement with previous research that did not see changes in DHA levels over the first two weeks (Yaqoob *et al.*, 2000; Faber *et al.*, 2011)

The use of a heterogeneous population of cells, such as white blood cells, has previously been discussed in Chapter 4. The research conducted within this chapter was also conducted using whole blood as the source of DNA and therefore this contained a mix of WBCs. Sorting of the cells using flow cytometry would have allowed for the analysis of individual sub-populations of WBCs, however this was not available within the existing laboratory facilities at Loughborough.

We did not include a control group within the current study in order to increase the number of participants who took the ω-3 PUFA supplement. The participants methylation levels at baseline were used to compare the methylation to in subsequent weeks. Therefore, this research has not measured if there is any variation within the methylation of the genes naturally over time. This could have been measured by including multiple baseline samples in order to assess habitual variation and have the ability to attribute any changes to the intervention.

A homogenous population of young males was used for this research in order to reduce confounding variables. This, however, limits the ability for these results to be generalised to the whole population. Multiple reasons contribute to the decision to include only male participants in the study. Firstly, there are significant differences in the levels of and the metabolism of fatty acids between males and females. Further to this there are differences in fatty acids between pre- and post-menopausal females.

Factoring in the menstrual cycle in studying females would have increased the time and cost of the present study. The current study has provided preliminary results into the investigation of fatty acids on inflammatory cytokine methylation. Secondly, due to the findings observed in Chapter 4 there was further justification in including only males into this preliminary work.

The participants included in this study were all healthy, free-living individuals. The lack of findings in this research may be due to the lack of inflammation within those individuals. Inducing inflammation or having an active inflammatory phenotype (e.g. as the result of a chronic disease) might alter the interaction between the fatty acids and the inflammatory mechanisms. Repetition of this study in a patient cohort (with for example COPD, obesity or diabetes) would provide important information into understanding the mechanisms of fatty acids on the epigenome.

The intervention period of this study was four weeks. It is possible that the failure to detect significant results may be related to this and that a longer intervention period, would have resulted in the epigenetic changes that this research sought to measure.

5.5 Conclusion

We conclude that altering the fatty acid profile of the blood through supplementation with ω-3 PUFAs over a four-week period does not impact the DNA methylation levels of specific CpG sites within the inflammatory cytokine encoding genes *TNF*, *IL6* and *IL1B*.

Chapter 6 The effects of eccentric exercise and fatty acid supplementation on the inflammatory response

6.1 Introduction

In Chapter 4 it was established that there are associations between blood fatty acid levels and DNA methylation of the *TNF* gene which encodes the inflammatory cytokine TNFα. Furthermore, it was then investigated whether dietary supplementation could influence the methylation profile of *TNF* and two other inflammatory cytokine genes, *IL6* and *IL1B* by changing the fatty acid composition of the blood and it was discovered that over a 28-day period of intervention no significant changes could be detected (Chapter 5). However, the lack of modification to the methylation profile that was observed may be because there were no inflammatory stimuli to activate these cytokine genes.

The research undertaken in this chapter seeks to establish whether the introduction of an inflammatory stimulus will provoke a change to gene methylation and hence expression, and therefore, it seeks to establish whether dietary supplementation will influence the outcome.

An inflammatory response can be stimulated by eccentric exercise (EE); (Peake, Nosaka and Suzuki, 2005). During an eccentric contraction a muscle lengthens, as opposed to shortening which occurs during a concentric contraction. The lengthening of the muscle under force causes disruption of sarcomeres in the myofibrils (Proske and Morgan, 2001). The force of the contraction and the length over which it is generated positively relate to the amount of disruption that occurs (Proske and Morgan, 2001). The greatest damage to the muscle as a result of eccentric contractions occurs during a large range of motion (Fochi *et al.*, 2016). In untrained/unaccustomed individuals, the EE induced muscle damage (EEIMD) causes increases in protein release from the muscle, local inflammatory response within the muscle (swelling, stiffness, infiltration of neutrophils) and a systemic inflammatory response evidenced by elevated levels of inflammatory cytokines, including IL6, TNFα,

IL1β, and the production of prostaglandins (Brown, Day and Donnelly, 1999; Clarkson and Hubal, 2002; Chen *et al.*, 2003). Therefore, EE was used to provide the desired inflammatory stimuli.

The inflammation triggered by EEIMD is known to be modulated by fatty acid supplementation (Jouris, McDaniel and Weiss, 2011; Tartibian, Maleki and Abbasi, 2011). Supplementation with ω-3 PUFAs for just seven days was shown to result in a decrease in localised soreness (inflammation) which had been induced by EE (Jouris, McDaniel and Weiss, 2011). Additional evidence supporting the influence of ω-3 PUFA on the inflammatory response is provided by a study in which a low dose ω-3 PUFA supplementation attenuated TNFα secretion, as well as that of prostaglandin E2 and lactate dehydrogenase (LDH). Serum levels of these inflammatory markers were seen to be reduced immediately after EE and the effect remained 48 hours later. The levels of the inflammatory markers, IL6 and creatin kinase (CK) responded in a like manner (Tartibian, Maleki and Abbasi, 2011).

Research to date has not established whether the inflammatory response to EEIMD is controlled through epigenetic mechanisms, such as DNA methylation, neither has it been established whether the observed attenuation of that response by ω-3 PUFA is the result of an epigenetic interaction with the fatty acid. To establish whether the induced inflammatory response is controlled by methylation, methylation levels will be measured alongside the cytokine expression of the same genes both before and after exercise, with and without ω-3 PUFA supplementation.

6.2 Methods

6.2.1 Ethical clearance

The local university ethics board approved the experimental procedure prior to recruitment. All participants provided written consent (example consent form in Appendix 2) after receiving verbal and written details of the experimental procedure, the intervention, and the risk involved.

6.2.2 Participants

Participants were recruited from the local area by word of mouth, advertisement posters, email and social media. Sixteen untrained, healthy, male participants (n=16) completed the intervention. In order ensure that only untrained individuals were recruited, participants were excluded if they had undertaken resistance training in the six months prior to the start of the study. In addition, it was a requirement that participants had not taken vitamin or ω-3 PUFAs supplementation during the previous six months. Other exclusion criteria included: any habitual use of anti-inflammatory drugs, a history of heart disease, coagulation/bleeding disorders or metabolic diseases, serious allergies, known blood borne virus, and those with current symptoms of general illness. Females were excluded from the study.

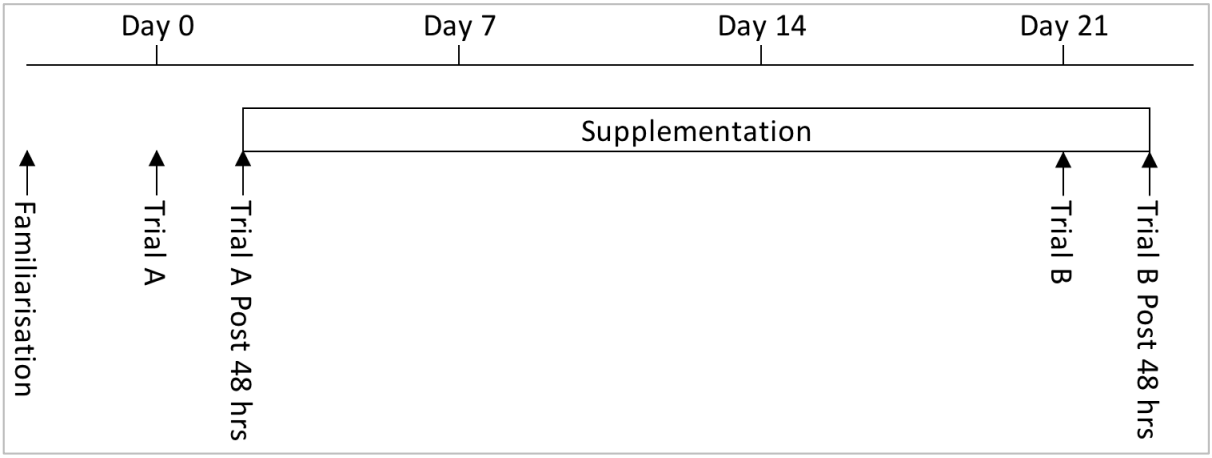


Figure 6-1 Intervention outline for five laboratory visits including two main experimental trial days separated by a 21-day supplementation period

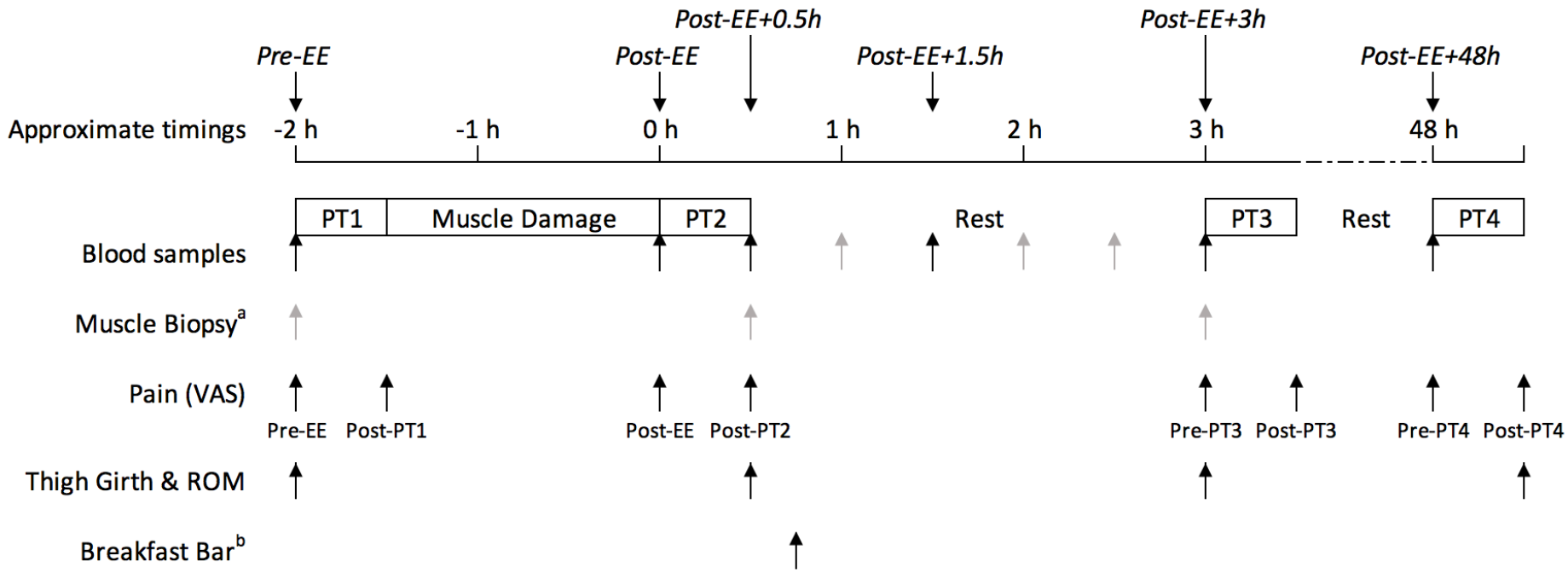


Figure 6-2 Study protocol and sampling procedure pre and post a bout of eccentric exercise. (a) muscle biopsies were optional; (b) standardised breakfast bar was provided to each participant after the second performance test. Range of motion (ROM), Visual analogue scale (VAS). Arrows in grey text indicate that samples collected but not analysed as part of this thesis.

6.2.3 Intervention outline

The study comprised two main experimental trial days (ETD). These were designated as Trial A and Trial B and were separated by three weeks double blind supplementation intervention. Participants attended the lab on five occasions; Familiarisation, Trial A, Trial A+48 hours, Trial B and Trial B+48 hours (see Figure 6-1). On each ETD various measurements and samples were taken as described in the following sections at these designated time points, see Figure 6-2;

1. Baseline before performance test one or eccentric exercise (Pre-EE)
2. After undertaking performance test 1 (Post-PT1)
3. Immediately after completing eccentric exercise (Post-EE)
4. After undertaking performance test 2 (Post-PT2)
5. 30 minutes after completing eccentric exercise (Post-EE+0.5h)
6. One and a half hours after completing eccentric exercise (Post-EE+1.5h)
7. Three hours after completing eccentric exercise (Post-EE+3h)
8. Before undertaking performance test 3 (Pre-PT3)
9. After undertaking performance test 3 (Post-PT3)
10. 48 hours after completing eccentric exercise (Post-EE+48h)
11. Before undertaking performance test 4 (Pre-PT4)
12. After undertaking performance test 4 (Post-PT4)

6.2.3.1 *Double-blind supplementation*

In order to maintain balanced intervention groups, participants were allocated into one of two groups on the basis of age, weight, height and preliminary force measurements. The diet of each participant was then supplemented, with either ω-3 PUFAs or with extra virgin olive oil (EVOO). Six capsules of either the PUFAs or the EVOO supplement were taken for 21 days, as shown in Figure 6-1. Consequently, the participants either received 3.0g EPA, 1.2g DHA and 0.9g of other ω-3 PUFAs (Table 6-1) or 6g of EVOO per day.

Table 6-1 Fatty acid composition of the omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplements (Norwegian Premium Omega-3 85%, Norwegian Pure-3 AS, Oslo, Norway)

Supplement contents (mg)	Per capsule	Per six capsules
Omega 3	850	5100
EPA	500	3000
DHA	200	1200
DPA and other ω-3 PUFAs	150	900
Total fat (mg)	1000	6000

eicosapentaenoic acid (EPA); docosapentaenoic acid (DPA); docosahexaenoic acid (DHA),

6.2.4 Familiarisation to experimental procedures

All participants attended a familiarisation session where they were fully informed of the study requirements and assessed for suitability to the study before they provided their written consent. Participants then completed a health screen questionnaire and an IPAQ questionnaire, before basic anthropometric measurements (height and weight) were taken. Further description of the experimental procedures and exercise testing were provided to the participants, including a description of all of the equipment and testing requirements.

Participants were individually fitted to the Humac Norm dynamometer (CSMI, Massachusetts, USA), and then given the opportunity to practice the movements required by completing the performance test described in Section 6.2.6.5. Verbal coaching on the required technique was provided as required.

6.2.5 Pre-trial standardisation

Participants were asked to refrain from strenuous exercise and the consumption of caffeine and alcohol during the 24 hours prior to attending the ETD's and until after they had completed the 48 hours post eccentric exercise lab visit. Starting 24 hours before Trial A, participants were asked to record a 3-day food diary detailing the food and drink they consumed during that period and they then used this diary to replicate the food consumed prior to and during Trial B. Participants were asked not to change their habitual diet or exercise routines for the duration of the study. On the morning of the ETD's, participants attended the lab having fasted for at least 12 hours and having consumed 500ml of water before arrival, water was then consumed *ad-libitum* throughout the day. Participants arrived at the lab for Trial A between 7am and 10am according to their chosen start time, and same start time was maintained for Trial B.

6.2.6 Experimental trial day

6.2.6.1 *Anthropometric measurements*

At the beginning of each ETD anthropometric measurements were recorded. Height was measured using a digital stadiometer to the nearest 0.1 centimetre (cm) and weight using digital weighing scales to the nearest 0.01 kilogram (kg). Body fat was estimated using bioelectrical impedance analysis (BIA).

6.2.6.2 *Thigh girth and range of motion*

Bilateral thigh girth was measured at the midpoint between the point just proximal to the patella and the inguinal crease at the hip. A surgical pen was used to mark the midpoint; this provided the measurement point for thigh girth measurements throughout the trial day. The measurement of thigh girth was taken whilst the participant was standing, relaxed and with all weight on that leg, three measurements were taken for each leg and an average recorded. Measurements were taken at four time points: Pre-EE, Post-PT2, Pre-PT3 and Pre-PT4. If the participant had consented to muscle biopsies (not analysed in this thesis), the measurements for this study were taken from the non-biopsied leg, which was always the leg that performed the eccentric exercise first.

Range of motion (ROM) of the hip and knee joints was measured using a Goniometer. For hip flexion, the participant lay supine on a firm bed, raised each leg unaided, keeping the leg straight and their back flat to the bed, measurements were taken with the centre of the Goniometer at the greater trochanter and when the leg reached its maximum flexion. Hip extension and knee flexion were measured with the participant lying prone. All the movements were voluntary, and no assistance was provided. Each leg was measured three times separately and an average of the measurements was recorded. Measurements were taken at the following time points: Pre-EE, Post-PT2, Pre-PT3 and Pre-PT4.

6.2.6.3 *Muscle soreness*

Participants were asked to indicate the level of muscle soreness using a visual analogue scale (VAS), which consisted of a 100mm line ranging from “No pain” to “Worst possible pain”. They recorded their pain perception by drawing a line on the scale whilst in a seated position with both legs fully extended horizontally in front of them. Measurements were taken at the following eight time points: Pre-EE, Post-PT1, Post-EE, Post-PT2, Pre-PT3, Post-PT3, Pre-PT4 and Post-PT4.

6.2.6.4 *Blood samples*

Venous blood samples were collected Pre-EE, and Post-EE, and then every 30 minutes for the next 3 hours and an additional sample 48 hours after exercise. To facilitate ease of sample collection and minimise the number of venepunctures, a catheter (Appleton Woods, Birmingham, UK) was inserted into the antecubital vein of the arm prior to exercise to obtain a baseline blood sample and samples throughout the trial day. The catheter was continually kept clear with isotonic saline solution (0.9% sodium chloride) administered approximately every 30 minutes. Post-EE+48h blood sample was taken via venepuncture. The samples used for further analysis were Pre-EE, Post-EE, Post-EE+0.5h, Post-EE+1.5h, Post-EE+3h and Post-EE+48h.

6.2.6.5 *Performance test*

Each performance test started with three (unless the final jump was highest to a maximum of five) countermovement jumps. Following this the participant completed a set of bilateral voluntary isometric, isokinetic concentric and isokinetic eccentric contractions on the dynamometer. Verbal encouragement and feedback were given throughout the performance test. Performance test results were not analysed as part of this thesis.

6.2.6.6 *Eccentric exercise protocol*

The dynamometer was used for the eccentric exercise protocol which consisted of 20 sets of maximal eccentric bilateral knee extensions; each set consisted of 10 repetitions with each set being separated by 1 minute's rest. The dynamometer lever arm was programmed to flex the participant's knee from a start position of 10° of flexion (where 0° is full extension, leg straight in seated position) to 90° of flexion. This allowed for an 80° range of motion. The participants began with their leg at the start position and were asked to maximally contract their quadriceps against a resistance while the lever arm moved to the finish position (90° knee flexion). Once at the finish position they were advised to relax their leg and the dynamometer moved them back to the start position to avoid a concentric contraction being performed. The lever arm moved at a set speed of $60° \cdot s^{-1}$. Visual feedback and verbal encouragement were provided to all participants to maximise torque output for each contraction.

6.2.6.7 *Plasma volume changes*

Changes in plasma were calculated using the method outlined by Dill and Costill (1974). Briefly, haematocrit (Hct) and haemoglobin (Hb) measured using a cell counter (Beckman Coulter Counter, USA) were used to calculate percentage changes in blood volume (BV), red cell volume (CV) and plasma volume (PV) using the following formulas:

$$BV_2 = BV_1 (Hb_1 / Hb_2)$$

$$CV_2 = BV_2 * Hct_2$$

$$PV_2 = BV_2 - CV_2$$

$$BV_{correction} = BV_2/BV_1$$

Subscripts 1 denotes Pre-EE blood sample and subscript 2 the Post-EE samples requiring plasma volume change. The $BV_{correction}$ value was used for subsequent plasma volume corrections.

6.2.7 Serum cytokines

At time points Pre-EE, Post-EE, Post-EE+0.5h, Post-EE+3h and Post-EE+48h, 10mL of blood was collected into vacutainers (Becton, Dickson & Company, UK) that contained no anticoagulant. The blood was allowed to clot at room temperature for 20 minutes and then centrifuged at 2800 rpm for 15 minutes at 4°C. The resulting serum was aliquoted and frozen -80°C for future analysis. Serum levels of IL1β, IL6 and TNFα cytokine levels were measured using BD™ Cytometric Bead Array (CBA) Enhanced Sensitivity Flex Sets (BD Bioscience, UK) on a flow cytometry platform (BD Accuri™ C6 Flow Cytometer, BD Bioscience, UK). Serum was diluted 1:3 with CBA buffer. The resulting values were corrected for plasma volume change, calculated in section 6.2.6.7.

Table 6-2 Assay statistics for the BD™ Cytometric Bead Array (CBA) Enhanced Sensitivity Flex Sets (BD Bioscience, UK) on a flow cytometry platform (BD Accuri™ C6 Flow Cytometer, BD Bioscience, UK)

Assay	Normal rages	Linear assay (R^2)	Limit of detection (pg/ml)	CV
Interleukin 1 beta	13-227pg/ml	0.9965	0.68	17.81
Interleukin 6	13-149pg/ml	1.0000	0.27	10.81
Tumour necrosis factor	42-203pg/ml	0.9999	0.28	33.44

normal ranges from (Sekiyama, Yoshiba and Thomson, 1994).

6.2.8 Gene expression

RNA was extracted from blood collected at time points Pre-EE, Post-EE, Post-EE+0.5h, Post-EE+1.5h, Post-EE+3h and Post-EE+48h. Promptly after blood collection, an aliquot of blood collected in EDTA-coated vacutainers was diluted 1:1 with deionised water. 250µL of diluted blood was mixed with TRIzol LS (TRIzol LS™, Invitrogen™, Thermo Fisher Scientific, USA) at a ratio of 1:3, mixed vigorously and allowed to stand at room temperature for five minutes to lyse the sample before being frozen at -20°C. Samples were transferred to storage at -80°C at the end of the ETD until purification.

RNA was extracted from the TRIzol using the Phenol-Chloroform method. Briefly, thawing the sample at room temperature for 10-15 minutes. Once completely thawed, 200µL of chloroform (pure, Sigma-Aldrich, UK) per 750µL of TRIzol used was mixed

with the homogenous sample. After thoroughly mixing the samples, the reaction was incubated for 15 minutes at room temperature. Following this, in order to separate out the phases, the samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase containing the RNA was removed to separate RNase free tubes and mixed with 2-propanol (pure, Sigma-Aldrich, UK) to precipitate the RNA (500µL per 750µL of TRIzol used). After incubating at room temperature for 10 minutes, the samples were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed to leave the RNA pellet, the pellet was then washed with 75% ethanol (Sigma-Aldrich, UK) with 1000µL per 750µL of TRIzol used and centrifuged at 7,500 x g for 5 minutes at 4°C. The excess supernatant was removed, pellet left to air dry for 10-20 minutes and resuspended in EDTA storage solution (Fisher Scientific, UK). The concentrations and purity of the RNA samples were assessed using UV spectroscopy (Nanodrop, Fisher Scientific, UK). Samples were considered to have sufficient purity when they had a A_{260}/A_{280} ratio of ≥ 1.7.

The extracted RNA was reverse transcribed using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, USA) according to the manufacturer's protocol to produce complementary DNA (cDNA) which was normalised to 5 ng/µL. Quantitative PCR (qPCR) was performed for each target gene using a Viia7 Real-Time PCR system (Applied Biosystems, USA). Each reaction contained 5 µL of SybrGreen PrecisionPlus qPCR Master Mix (PrimerDesign, UK), 0.5 µL of forward and reverse primer and 4 µL of 5 ng/µL cDNA. All samples were run in duplicate using the following cycling conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Melt curves were visually inspected for a single peak indicating the generation of a single product. The relative mRNA expression of the genes of interest were calculated using the $\Delta\Delta$Ct formula; relative to the expression of GAPDH "Glyceraldehyde-3-phosphate dehydrogenase" and using the average pre-exercise Ct for Trial A from each supplement group as the second reference. Data is presented as fold change mean ± standard deviation.

Table 6-3 PCR primer sequences

Gene ID	Primer	Sequence
DNMT1	Forward	5'-TACCTGGACGACCCTGACCTC-3'
	Reverse	5'-CGTTGGCATCAAAGATGGACA-3'
DNMT3a	Forward	5'-TATTGATGAGCGCACAAGAGAGC-3'
	Reverse	5'-GGGTGTTCCAGGGTAACATTGAG-3'
DNMT3b	Forward	5'-GGCAAGTTCTCCGAGGTCTCTG-3'
	Reverse	5'-TGGTACATGGCTTTTCGATAGGA-3'
PPARGC1A	Forward	5'-CAGCCTCTTTGCCCAGATCTT-3'
	Reverse	5'-TCACTGCACCACTTGAGTCCAC-3'
IL6	Forward	5'-GCAGAAAAAGGCAAAGAATC-3'
	Reverse	5'-CTACATTTGCCGAAGAGC-3'
TNF	Forward	5'-AGGCAGTCAGATCATCTTC-3'
	Reverse	5'- TTATCTCTCAGCTCCACG-3'
GAPDH	Forward	5'- GCCTCAAGATCATCAGCAATGCCT-3'
	Reverse	5'- TGTGGTCATGAGTCCTTCCACGAT-3'

6.2.9 DNA methylation

Blood samples were collected into EDTA-coated vacutainers and frozen promptly on the trial day at -20^{o}C before being transferred to -80^{o}C for long term storage prior to DNA extraction. DNA was extracted from blood and bisulphite-converted using EpiTect LyseAll kit (Qiagen, Germany). DNA methylation levels of *TNF*, *IL1B* and *IL6* were measured by pyrosequencing using the PyroMark Q48 Autoprep (Qiagen, Germany) with the assays designed and validated in Chapter 3. Data is presented as mean ± standard deviation.

6.2.10 Statistics

Descriptive statistics were calculated prior to the removal of outliers and replacement of missing values. Outliers were classed as data points three times the interquartile range above the upper quartile and below the lower quartile. These data points were removed prior to analysis. Due to the exclusion of cases in a listwise manner during analysis of variants (ANOVA), missing values were replaced using the multiple imputation method. The effect of variables on each other was unknown at this point and therefore all variables (split into two groups, see below) were used in the model. Five imputations were pooled to give a variable to use for further analysis.

- Anthropometrics, blood measures (Methylation, RNA expression, Protein)
- VAS, thigh girth, ROM

A three-way mixed between-within-within (BWW) ANOVA was used to understand the effects of supplement (EVOO or ω-3 PUFAs), trial (Trial A and Trial B), and time point (including where data available: Pre-EE, Post-EE, Post-EE+0.5h, Post-EE+1.5h, Post-EE+3h and Post-EE+48h) on each analysed variable. Distribution of the data was assessed by Shapiro-Wilk's test, due to violations being observed in only a few instances and ANOVAs being considered as fairly robust, violations were noted but the tests were performed on non-transformed data. Due to the small sample size, the Greenhouse-Geisser correction was used for all ANOVAs performed.

6.3 Results

6.3.1 Baseline measurements

The baseline characteristics of the participants are presented in Table 6-4, and comparisons between their baseline blood measurements are shown in

Table 6-5. Prior to intervention there were no significant differences between the characteristics of the two groups. The baseline blood measurements also indicated that there was no significant difference in measured blood variables between the groups with the exception of serum IL6 cytokine levels which were higher at baseline in the group which was to be supplemented with ω-3 PUFA (p = 0.033).

The EE performed was designed to cause maximum muscle fatigue and damage without injuring the participants. However, there were two instances where the participant was not able to complete the eccentric exercise protocol on both legs. One participant (in the EVOO group) only completed one leg during Trial B; and one participant from the ω-3 PUFA group only completed one leg for both Trial A and B. Due to the limited size of the cohort, it was decided that the results of these individuals would be included in the analysis.

Table 6-4 Participant characteristics as baseline split by intervention group

Descriptive	Leg*	EVOO (n = 8)	ω-3 PUFAs (n = 8)	*p*
Age (years)		28.5 ± 11.2	26.7 ± 7.1	0.703
IPAQ		2,577 ± 1,874	2,892 ± 2,289	0.768
Height (cm)		179.5 ± 3.8	180.6 ± 6.5	0.699
Weight (Kg)		80.5 ± 10.7	83.5 ± 19.5	0.707
Body Fat (Kg)		16.4 ± 7.5	19.7 ± 12.4	0.520
Body Fat (%)		19.8 ± 6.9	21.7 ± 9.9	0.657
Water Weight (%)		58.1 ± 4.8	56.6 ± 6.9	0.635
BMI (m/kg^2)		25.0 ± 3.3	25.5 ± 5.2	0.814
Pain (VAS, mm)		12 ± 10	10 ± 7	0.675
Knee Flexion (degrees)	First leg	121 ± 6	120 ± 7	0.886
	Second leg	122 ± 6	121 ± 7	0.740
Hip Flexion (degrees)	First leg	69 ± 9	64 ± 10	0.336
	Second leg	67 ± 10	68 ± 12	0.804
Hip Extension (degrees)	First leg	22 ± 7	21 ± 5	0.929
	Second leg	22 ± 7	19 ± 5	0.348
Thigh Girth (degrees)	First leg	55 ± 6	55 ± 6	0.819

* Leg which performed the eccentric exercise first or second. Data presented mean ± standard deviation. T-test probability value comparing Extra virgin olive oil (EVOO) and omega-3 polyunsaturated fatty acids (ω3PUFA) groups.

Table 6-5 Baseline blood analysis comparisons between EVOO and ω-3 PUFA groups

Variable	EVOO group (n = 8)	ω-3 PUFAs group (n = 8)	*p*
White Blood Cell (WBC) Counts (10^9/L) [range*]			
Total WBC [3.5-10.0]	4.85 ± 0.99	4.86 ± 0.74	0.973
Neutrophils [1.6-7.0]	2.90 ± 1.09	2.46 ± 0.57	0.330
Monocytes [0.2-0.8]	0.51 ± 0.15	0.45 ± 0.11	0.345
Basophils [0.0-0.2]	0.09 ± 0.04	0.08 ± 0.05	0.554
Eosinophils [0.0-0.5]	0.16 ± 0.05	0.18 ± 0.15	0.828
Lymphocytes [1.0-3.0]	1.61 ± 0.43	1.71 ± 0.22	0.563
DNA Methylation (%) - Interleukin 1 beta (*IL1B*)			
CpG1	89.5 ± 2.7	90.2 ± 1.1	0.535
CpG2	94.4 ± 0.8	94.3 ± 0.7	0.949
CpG3	86.2 ± 3.1	87.5 ± 2.2	0.345
CpG4	90.4 ± 2.3	91.6 ± 1.9	0.273
CpG5	74.6 ± 1.7	74.9 ± 1.5	0.701
CpGavg	87.0 ± 2.0	87.7 ± 1.3	0.409
DNA Methylation (%) - Interleukin 6 (*IL6*)			
CpG1	91.5 ± 2.3	90.5 ± 1.3	0.336
CpG2	91.8 ± 0.8	92.1 ± 1.6	0.596
CpG3	92.3 ± 0.8	93.0 ± 1.6	0.334
CpG4	88.8 ± 0.7	88.6 ± 1.2	0.735
CpG5	83.9 ± 0.8	83.8 ± 1.7	0.958
CpG6	89.6 ± 1.3	89.9 ± 0.8	0.575
CpGavg	89.7 ± 0.6	89.7 ± 1.0	0.905
DNA Methylation (%) - Tumor necrosis factor (*TNF*)			
CpG1	8.4 ± 1.6	7.9 ± 1.0	0.464
CpG2	5.9 ± 1.9	6.8 ± 1.0	0.242
CpG3	6.7 ± 2.3	6.9 ± 0.8	0.853
CpG4	11.2 ± 3.1	11.5 ± 1.5	0.864
CpGavg	8.1 ± 2.1	8.2 ± 1.0	0.836
Serum Cytokines (pg/ml)			
Tumor necrosis factor alpha (TNFα)	195.7 ± 163.0	271.7 ± 223.9	0.451
Interleukin 6 (IL6)	551.2 ± 223.0	294.5 ± 218.8	**0.036**
Muscle Damage Markers (U/l)			
Lactate dehydrogenase (LDH)	254.9 ± 130.6	261.2 ± 77.3	0.909
Creatin kinase (CK)	147.6 ± 27.1	169.9 ± 30.3	0.143
Myoglobin	34.7 ± 9.7	44.2 ± 21.8	0.285

Data presented mean ± standard deviation. T-test probability value comparing Extra virgin olive oil (EVOO) and omega-3 polyunsaturated fatty acids (ω-3 PUFA) groups. *normal range quoted from the coulter counter data sheet.

6.3.2 Perception of pain (visual analogue scale)

The participant's perception of pain in both their legs was assessed using a VAS scale. The results are summarised in Table 6-6 and are depicted graphically in Figure 6-3. It was found that the level of pain perceived by the participants increased significantly after completing the EE. Their perceived level of pain remained elevated after EE until at least 48 hours later when the last measurement was recorded for the trial.

There was no statistically significant three-way interaction between the two trials, time and supplements (trial x time x supplement), F (3.953,55.345) = 1.388, p = 0.250, partial η^2 = 0.090.

The two-way interaction (trial x time) was marginally not significant in the olive oil group F(3.486,24.403) = 2.332, p = 0.091, partial η^2 = 0.250, with the tendency for lower perceived pain after EVOO supplementation. There was no two-way interaction (trial x time) in the ω-3 PUFA group.

Table 6-6 Perception of pain measured using a visual analogue scale (VAS).

Supplement	Trial	Pre-EE	Post-PT1	Post-EE	Post-PT2	Pre-PT3	Post-PT3	Pre-PT4	Post-PT4	*p* simple interaction $	*p* two-way #	*p* three-way §
EVOO	A	12 ± 10 ^{a}	25 ± 11 ^{a,b}	66 ± 25 ^{b,c}	54 ± 17 ^{b,c}	41 ± 13 ^{a,b,c}	52 ± 19 ^{b,c}	59 ± 22 ^{c}	58 ± 18 ^{c}	<0.001	0.091	0.250
	B	7 ± 6 ^{a,c}	17 ± 9 ^{a,b}	55 ± 29 ^{a,c,d}	55 ± 28 ^{c,d}	35 ± 18 ^{a,c,d}	38 ± 17 ^{b,d}	47 ± 22 ^{d}	41 ± 21 ^{a,c,d}	0.001		
ω3PUFA	A	10 ± 7 ^{a}	26 ± 14 ^{a,b}	57 ± 24 ^{c}	60 ± 20 ^{C}	48 ± 23 ^{b,c}	60 ± 18 ^{c}	55 ± 35 ^{a,c}	58 ± 23 ^{c}	<0.001	0.471	
	B	8 ± 6 ^{a}	20 ± 8 ^{a}	62 ± 20 ^{b}	64 ± 16 ^{B}	52 ± 19 ^{b}	60 ± 16 ^{b}	58 ± 26 ^{b}	58 ± 23 ^{b}	<0.001		

Pre and post eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented in millimetres (mm) as mean ± standard deviation. Means (within trial) not sharing a letter (a,b,c,d) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

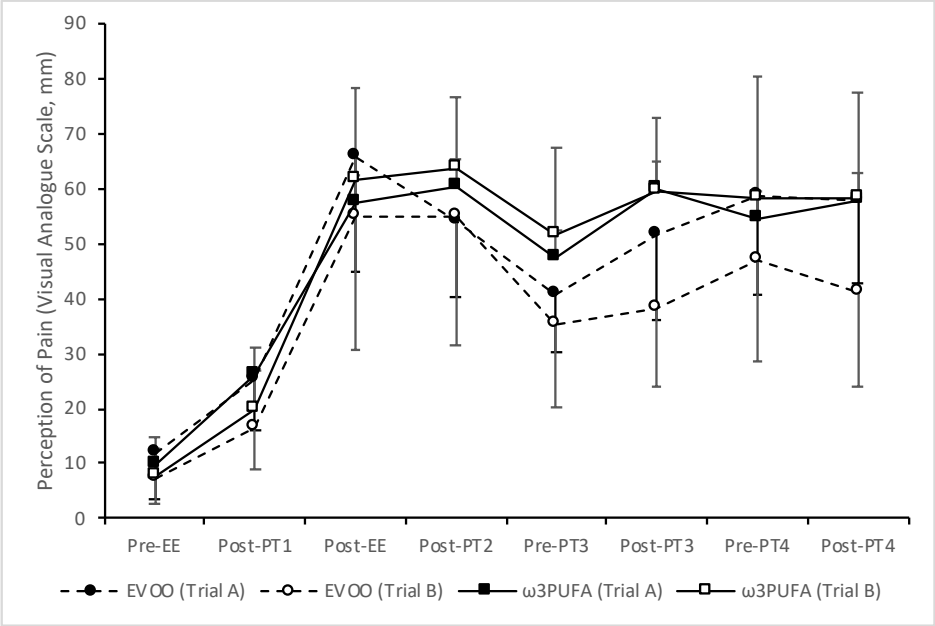


Figure 6-3 Perception of pain measured using a visual analogue scale (VAS) pre and post eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± 95% confidence intervals.

6.3.3 Swelling (thigh girth)

Changes in thigh girth, used as an indicator of swelling, were not detected. The results are presented graphically in Figure 6-4. There was no difference over time between trials (two-way interaction: time x trial, within supplementation group) or between supplementation group (three-way interaction: time x trial x supplementation). There was a small increase in thigh girth of the ω-3 PUFA group during Trial B between Pre-EE and the Post-EE+48h (56.0 ± 6.3 vs. 56.4 ± 6.3, p = 0.029) after correction for multiple testing.

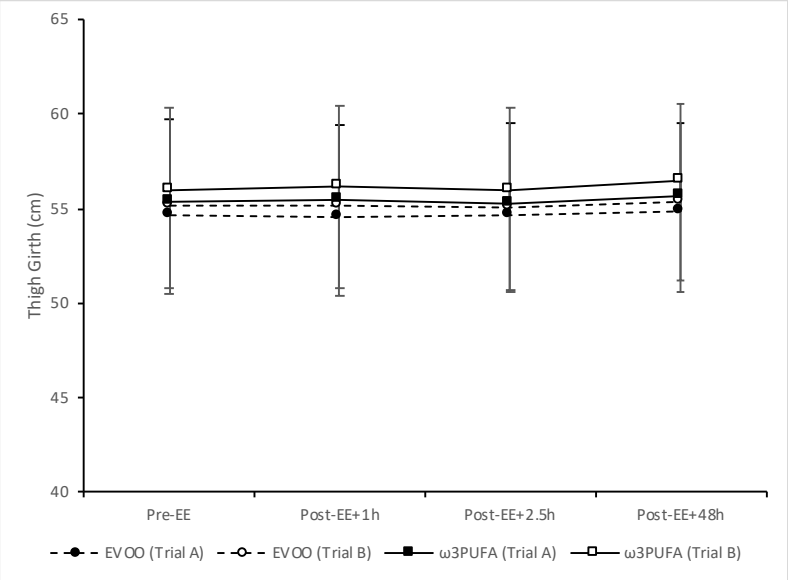


Figure 6-4 Thigh girth measurements pre and post eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± 95% confidence intervals.

6.3.4 Range of motion (knee and hip joints)

We found that the ROM around hip joint for the first leg undertaking the EE was reduced following the exercise but that this reduction became less apparent over time such that it had mostly returned to baseline values 48 hours after the exercise, as shown in Figure 6-5. This was the case in both groups with no effect seen as the result of the intervention, see Table 6-7. This trend was not as pronounced in the leg that had performed the EE second.

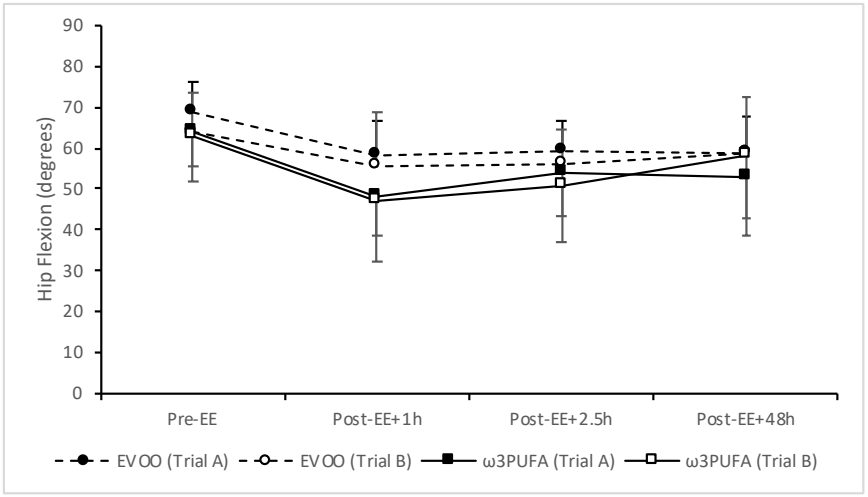
In Trial A it was found that the knee flexion ROM for the first leg undertaking the EE was reduced following the exercise, with no recovery to pre-exercise levels within the 48 hours after exercise. However, after supplementation (i.e. Trial B) no significant decreases in ROM were observed. The interaction (time x trial) was calculated and found to be significant within the EVOO group ($F(2.144,15.009) = 6.808$, $p = 0.007$, partial $\eta^2 = 0.493$) but not in the ω-3 PUFA group. The results for the second leg undertaking the exercise displayed the same trend as the first leg but again were not as pronounced.

Table 6-7 Range of motion around the hip and knee joints before and after an intense bout of eccentric exercise and the effect of EVOO or ω3 PUFA supplementation

Movement	Leg *	Supplement	Trial	Pre EE	Post EE+1h	Post EE+2.5h	Post EE+48h	p simple interaction $	p two-way #	p three-way §
Hip Flexion	First	EVOO	A	69 ± 9 a	58 ± 10 b	59 ± 9 b	59 ± 11 b	0.001	0.432	0.687
			B	64 ± 11 a	55 ± 16 a,b	56 ± 10 b	59 ± 16 a,b	0.096		
		ω3PUFA	A	64 ± 10 a	48 ± 12 b	54 ± 13 b	53 ± 18 a,b	0.002	0.106	
			B	63 ± 13 a,c	47 ± 18 b	51 ± 17 b,c	58 ± 18 c	0.001		
	Second	EVOO	A	67 ± 10 a	60 ± 11 a,b	58 ± 9 b	57 ± 13 a,b	0.022	0.175	0.141
			B	62 ± 14	55 ± 18	57 ± 13	57 ± 15	0.002		
		ω3PUFA	A	68 ± 12 a	51 ± 10 b	57 ± 12 b	58 ± 16 a,b	0.169	0.119	
			B	62 ± 13 a	54 ± 14 b	53 ± 12 b	60 ± 13 a,b	<0.001		
Hip Extension	First	EVOO	A	22 ± 7 a	18 ± 7 a,b	18 ± 5 b	19 ± 7 a,b	0.008	0.262	0.864
			B	19 ± 6	17 ± 7	17 ± 6	16 ± 6	0.152		
		ω3PUFA	A	21 ± 5	17 ± 5	18 ± 7	19 ± 6	0.116	0.671	
			B	19 ± 6	17 ± 6	17 ± 5	17 ± 6	0.191		
	Second	EVOO	A	22 ± 7 a	18 ± 7 a,b	17 ± 6 b	18 ± 8 a,b	0.008	0.283	0.370
			B	20 ± 7 a	17 ± 8 a,b	16 ± 7 b	18 ± 7 a	0.306		
		ω3PUFA	A	19 ± 5	19 ± 4	18 ± 7	17 ± 6	0.008	0.226	
			B	19 ± 5	16 ± 6	16 ± 6	18 ± 5	0.125		
Knee Extension	First	EVOO	A	121 ± 6 a	116 ± 5 b	115 ± 4 b	113 ± 8 b	<0.001	0.007	0.729
			B	121 ± 5	115 ± 6	116 ± 5	118 ± 7	0.158		
		ω3PUFA	A	120 ± 7 a	114 ± 9 b	112 ± 8 b	112 ± 17 a,b	0.006	0.481	
			B	118 ± 7	114 ± 4	114 ± 7	115 ± 9	0.123		
	Second	EVOO	A	122 ± 6 a	118 ± 5 b	118 ± 4 a,b	117 ± 6 a,b	0.035	0.836	0.611
			B	121 ± 6	117 ± 6	118 ± 5	117 ± 8	0.157		
		ω3PUFA	A	121 ± 7	116 ± 6	118 ± 5	117 ± 8	0.088	0.496	
			B	119 ± 7	116 ± 7	117 ± 7	118 ± 8	0.301		

Measurements taken pre and post eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. * Leg which performed the eccentric exercise first or second. Data presented in degrees as mean ± standard deviation. Means not sharing a letter (a,b,c) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

a)



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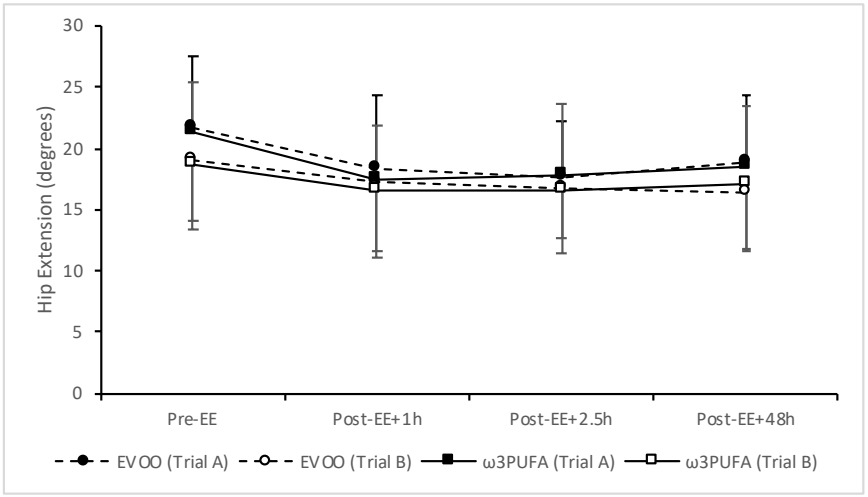

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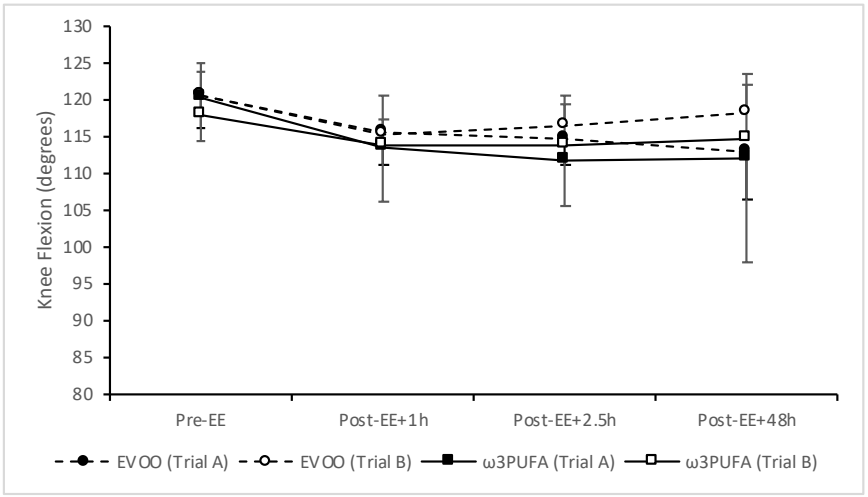


Figure 6-5 Range of motion for hip flexion (a), hip extension (b), knee flexion (c) pre- and post -eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. For the first leg to perform the EE. Data presented as mean ± 95% confidence intervals.

6.3.5 Muscle damage-related inflammatory markers in the serum

The results for the 3 serum muscle damage markers, creatin kinase (CK), lactate dehydrogenase (LDH) and myoglobin (Mb) that were measured are depicted in Table 6-8 and Figure 6-6. Note that the x axis in the graphical representations (Figure 6-6 a) are not to scale and so the rate of changes apparent here are not representative.

During Trial A the levels of CK were seen to increase between baseline and post-EE and then to continue to rise. The differences between time points were statistically significant for both groups (p<0.002), which at this this point had not received supplemention and therefore effectively homogenous. Post-supplementation (Trial B), the levels of CK were also seen to increase as a result of the EE and to continue to rise thereafter. Statistical analysis confirms that the visualised trend was significant for both EVOO and ω-3 PUFA (p<0.006). The Pre-EE levels of Trial B were similar to those of pre-supplementation (Trial A) but the observed increase was less than Trial A. This difference in trend pre- and post-supplementation was significant for both EVOO and ω-3 PUFA (p<0.062), however, as discussed below, this may not have been the result of the supplementation itself. The three-way ANOVA indicated that there was no significant interaction between supplement type, trial day and time point (p = 0.781).

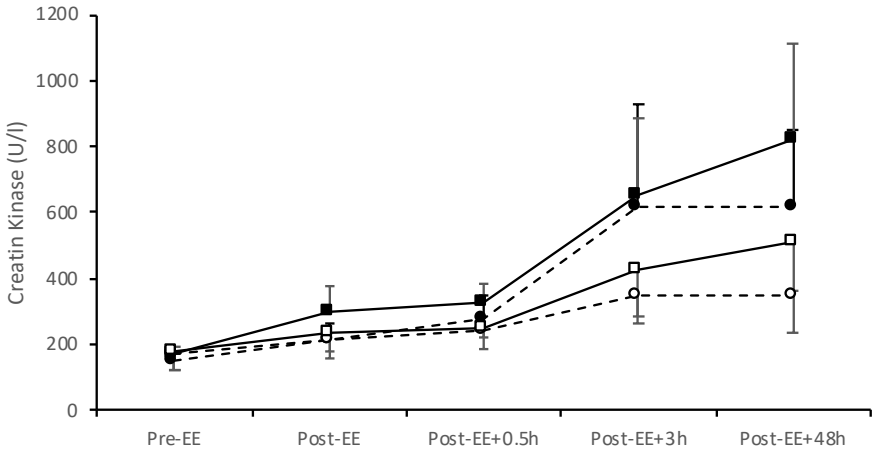
No statistically significant changes were observed in the levels of LDH in either group prior to supplementation, i.e. during the first trial day (p=>0.155). Neither were there any significant changes observed for levels of this marker in the EVOO supplemented group post-supplementation (p=>0.521). However, there was a statistically significant, but small, increase in the levels of LDH during Trial B for the group which had been supplemented with ω-3 PUFA. In neither group was there an interaction between pre and post supplementation (p=>0.565). The levels of Mb pre-EE were consistent between the groups and on both trial days during which, for both groups, in response to EE the levels of this marker increased over the following 3 hours but had returned to pre-exercise levels when measured 48 hours later. This observed trend was significant (p =<0.015). Post-supplementation the increase in levels of Mb were not as pronounced but the trend was the same, with levels increasing after exercise and having returned to pre-exercise levels 48 hours later. There was no statistical interaction between the supplementation groups (p=0.497).

Table 6-8 Muscle damage markers in serum

			Pre-EE	Post-EE	Post-EE+0.5h	Post-EE+3h	Post-EE+48h	p simple interaction $	p two-way #	p three-way §
CK	EVOO	Trial A	147.6 ± 27.1 ^{a}	214.1 ± 59.1 ^{b}	275.3 ± 90.1 ^{c}	618.2 ± 370.9 ^{a,b,c,d}	616.5 ± 275.0 ^{d}	**0.002**	**0.009**	0.781
		Trial B	173.6 ± 58.2 ^{a}	213.6 ± 66.5 ^{a}	243.6 ± 68.1 ^{a}	348.9 ± 106.4 ^{B}	351.3 ± 135.7 ^{a,b}	**0.006**		
	ω3PUFA	Trial A	169.9 ± 30.3 ^{a}	300.2 ± 93.6 ^{a,b,c}	324.7 ± 69.3 ^{b,d}	652.0 ± 276.4 ^{c,d}	818.3 ± 352.2 ^{d}	**<0.001**	0.062	
		Trial B	178.9 ± 69.2 ^{a}	236.8 ± 73.7 ^{b}	252.2 ± 34.8 ^{a,b,c}	428.2 ± 173.3 ^{c,d}	507.6 ± 175.0 ^{d}	**<0.001**		
LDH	EVOO	Trial A	254.9 ± 130.6	255.9 ± 61.5	258.4 ± 73.0	306.9 ± 81.2	258.7 ± 52.1	0.365	0.579	0.497
		Trial B	271.8 ± 169.2 ^{a,b}	231.2 ± 61.6 ^{a}	260.0 ± 58.7 ^{a,b}	271.8 ± 68.5 ^{B}	265.6 ± 64.7 ^{b}	0.521		
	ω3PUFA	Trial A	261.2 ± 77.3	270.4 ± 66.9	281.8 ± 59.8	294.6 ± 67.0	328.9 ± 100.2	0.155	0.565	
		Trial B	246.6 ± 68.8 ^{a,c}	252.8 ± 73.1 ^{a}	261.2 ± 81.0 ^{a,c}	288.3 ± 78.1 ^{B}	289.0 ± 90.4 ^{b,c}	**<0.001**		
Mb	EVOO	Trial A	34.7 ± 9.7 ^{a}	200.0 ± 116.5 ^{b}	342.1 ± 226.3 ^{a,b}	498.8 ± 412.8 ^{a,b}	63.1 ± 54.4 ^{a,b}	**0.015**	**0.038**	0.497
		Trial B	33.0 ± 10.5 ^{a}	96.1 ± 41.8 ^{b}	151.3 ± 69.0 ^{c,d}	168.3 ± 76.3 ^{D}	36.0 ± 11.4 ^{a}	**<0.001**		
	ω3PUFA	Trial A	44.2 ± 21.8 ^{a}	247.1 ± 142.3 ^{b}	401.4 ± 288.7 ^{a,b}	446.8 ± 345.3 ^{a,b}	53.6 ± 15.2 ^{a,b}	**0.008**	**0.023**	
		Trial B	36.8 ± 10.8 ^{a}	118.0 ± 54.7 ^{b}	197.5 ± 108.0 ^{c}	220.7 ± 124.4 ^{b,c}	39.7 ± 10.5 ^{a}	**0.002**		

Serum creatin kinase (CK), lactate dehydrogenase (LDH) and myoglobin (Mb) (U/l) measurements from pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± standard deviation. Means not sharing a letter (a,b,c,d) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

a)



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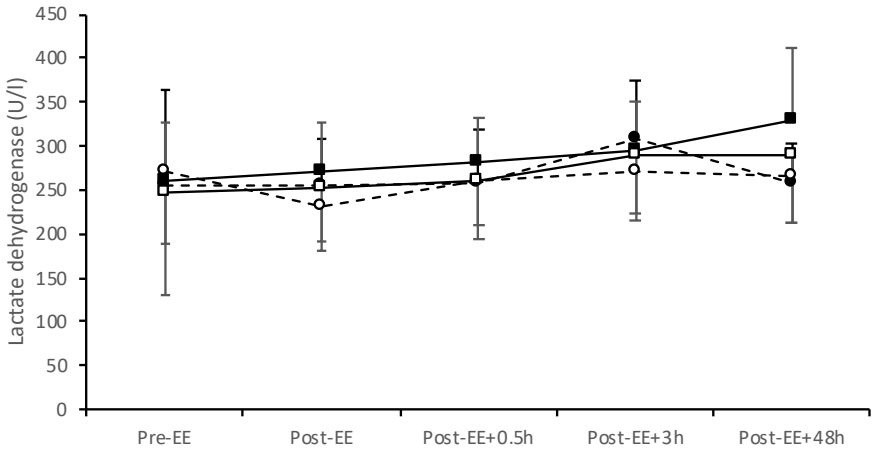

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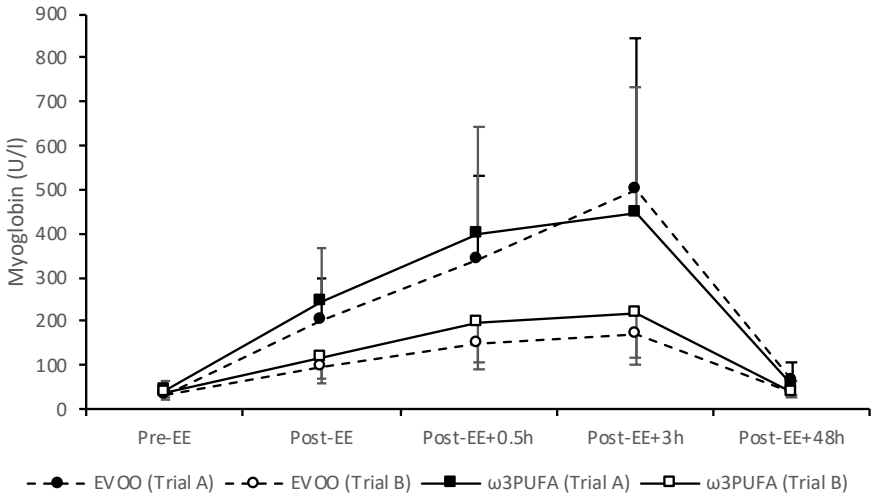


Figure 6-6 Serum muscle damage markers: Creatin kinase (a), Lactate dehydrogenase (b), Myoglobin (c) pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA). Data presented as mean ± 95% confidence intervals.

6.3.6 Serum cytokines

Measurements of the serum cytokines IL6 and TNFα are summarised in Table 6-9 and discussed below.

The pre-supplementation Trial A results showed that serum IL6 cytokine levels increased after EE, with the highest measurement occurring at the Post EE+0.5hr time point. The results for the ω-3 PUFA group were significant (p=0.004) whilst those for the EVOO were not (p=0.069). There was a large range in data values and this was responsible for the failure to show significance. However, at the time of Trial A the supplementation intervention had not yet taken place and therefore it is also valid to consider the cohorts as a single population. The combined results show that the increase in IL6 after EE was indeed significant (F(3.017,45.249)=9.559,p<0.001).

In respect of the group who had received the EVOO supplement the post-supplementation Trial B IL6 measurements displayed a similar but non-significant (p=0.102) and a less pronounced trend to that observed pre-supplementation. The results for the group supplemented with ω-3 PUFA also displayed a similar less pronounced trend post-supplementation and this was also not significant (p=0.062). There was no interaction between the two supplementation groups with respect to time points and trials (p=0.890).

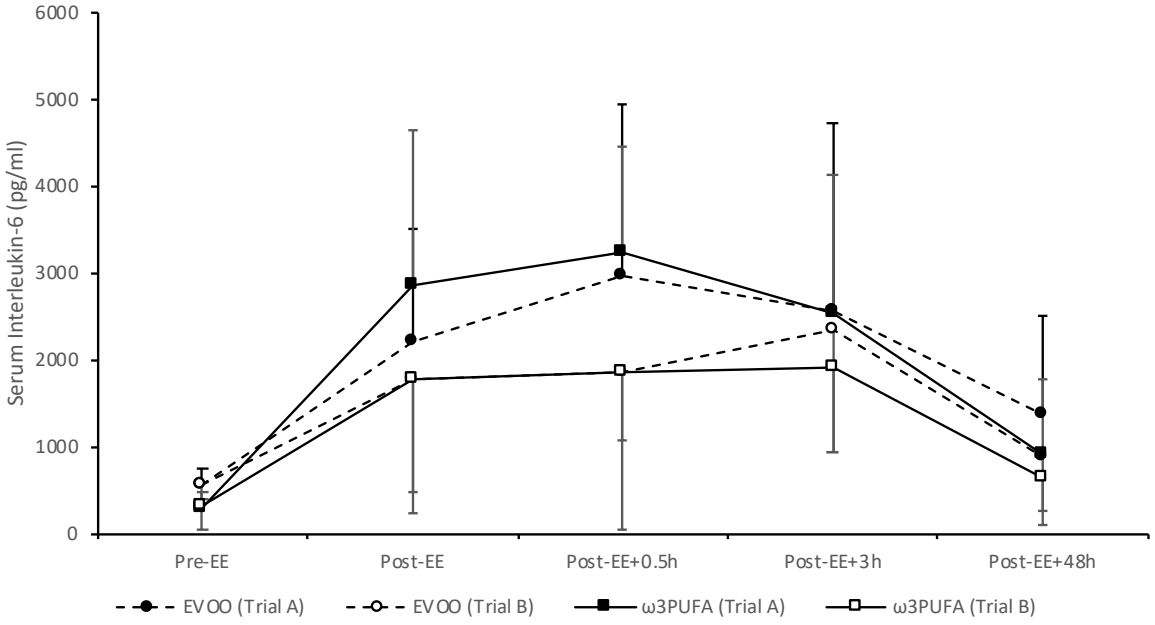
The pre-supplementation Trial A results showed that serum TNFα cytokine levels did not increase immediately following EE but did subsequently rise and the highest values recorded were at the Post-EE+3h time point and that they had fallen but not fully returned to Pre-EE levels after 48 hours. The results for the two groups both showed this trend but only the results for the EVOO designated group were significant (p=0.027). The combined results however did confirm that the increase was significant F(2.479,37.034)=5.742, p=0.004. Post-supplementation results showed similar trends for both groups but due to the small sample sizes and large variation in levels between participants the results were not significant (p=0.85 for EVOO and p=0.29 for ω-3 PUFA). Again, there was no interaction between the two supplementation groups with respect to time points and trials (p=0.540).

Table 6-9 Serum cytokine levels in response to eccentric exercise

Serum Cytokine	Supplement	Trial	Pre-EE	Post-EE	Post-EE+0.5h	Post-EE+3h	Post-EE+48h	p simple interaction $	p two-way #	p three-way §
IL6 (pg/ml)	EVOO	A	551.2±223.0	2,198.2±1,548.7	2,959.0±2,368.4	2,552.9±2,585.5	1,361.6±1,366.1	0.069	0.645	0.890
		B	568.5±198.4	1,762.2±1,538.7	1,846.6±2,152.7	2,346.1±1,680.5	887.2±762.2	0.102		
	ω3PUFA	A	294.5±218.8 a	2,851.1±2,148.6 a,b	3,238.4±1,436.1 b	2,539.2±1,884.8 a,b	912.3±1,039.4 a,b	**0.004**	0.343	
		B	326.4±330.3 a	1,762.3±1,844.7 a,b	1,842.7±926.2 b	1,895.9±1,140.7 a,b	635.7±651.4 a,b	0.062		
TNFα (pg/ml)	EVOO	A	195.7±163.0	264.6±121.2	232.2±94.1	496.5±248.3	272.0±138.1	**0.027**	0.104	0.540
		B	230.7±155.6	276.8±190.9	289.6±239.6	253.7±128.0	241.3±64.9	0.850		
	ω3PUFA	A	271.7±223.9	266.9±179.3	218.6±110.2	456.3±216.9	346.6±145.7	0.192	0.536	
		B	293.4±256.9	174.4±91.9	166.1±100.0	324.6±196.6	309.9±307.1	0.290		

Serum interleukin-6 (IL6) and tumor necrosis factor alpha (TNFα) cytokine levels pre and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± standard deviation. Means not sharing a letter (a,b) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

a)



b)

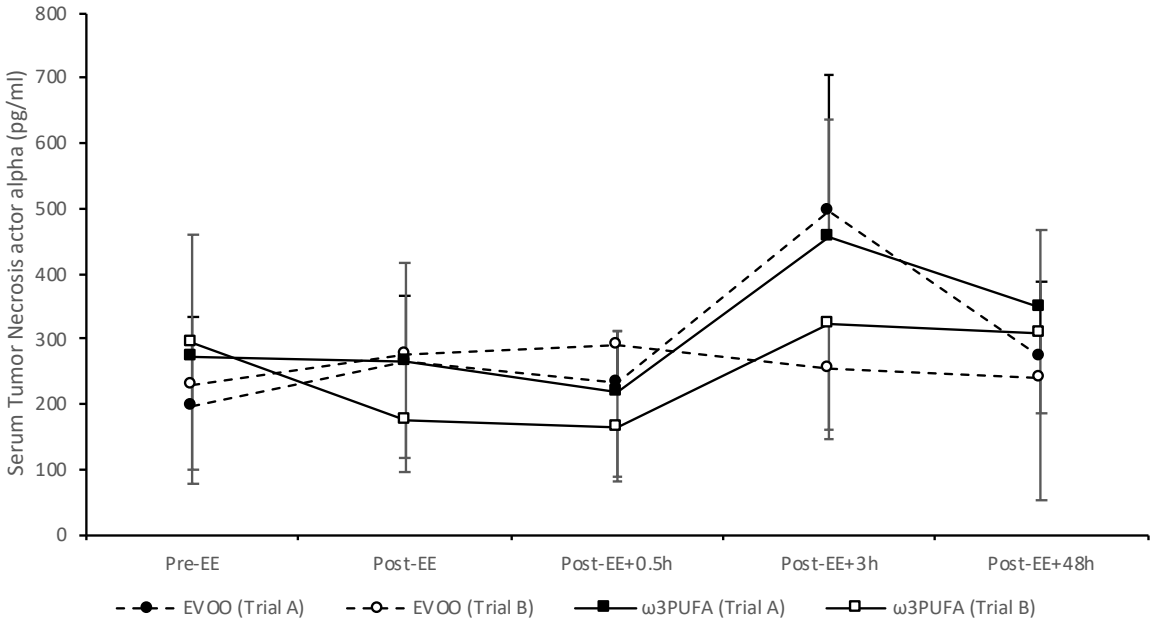


Figure 6-7 Serum cytokine levels: Interleukin-6 (a), Tumor necrosis factor alpha (b) - pre and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA). Data presented as mean ± 95% confidence intervals.

6.3.7 Gene expression

The results of gene expression have been presented as fold increase and are set out in Table 6-10. The expression of seven genes was measured. Following EE before supplementation (Trial A) results for the combined groups showed that the expression of COX2 was seen to increase, and that of DNMT3b, IL6 and TNF to decrease. The differential expressions across the time points of Trial A for these genes were significant (one-way repeated measures ANOVA). The changes observed in COX2, and IL6 expression were not significant after correction for multiple testing. Expression of DNMT3b was significantly lower at post-EE+1.5h and +3h (p=<0.014). The expression of TNF post-EE+0.5h was significantly lower than immediately after the exercise (p=0.036).

After supplementation, Trial B, the two groups are considered separately. No any significant results were evident after correction for multiple testing except as regards DNMT3b and IL1B in the group that had been supplemented with ω-3 PUFA. Expression of DNMT3b was observed to decrease after EE and expression of IL1B was observed to increase after EE.

As regards to the expression of DNMT3b, a significant three-way interaction (time x trial x supplementation) F(3.723,52.128)=3.299, p=0.02 was observed. Furthermore, the two-way interaction (time x trial) for the ω-3 PUFA group was significant (p=0.021) as a result of a small decrease in expression immediately after EE in Trial B compared to the increase observed in Trial A.

Table 6-10 Gene expression

Gene	Supplement	Trial	Pre-EE	Post-EE	Post-EE+0.5h	Post-EE+1.5h	Post-EE+3h	Post-EE+48h	p simple interaction $	p two-way #	p three-way §
COX2	EVOO	A	1.1 ± 0.5	1.5 ± 0.8	1.5 ± 0.8	1.2 ± 0.3	1.3 ± 0.7	1.1 ± 0.7	0.183	0.380	0.673
		B	0.7 ± 0.4	1.5 ± 0.7	1.5 ± 0.9	1.4 ± 0.6	1.3 ± 0.6	0.9 ± 0.3	**0.030**		
	ω3PUFA	A	1.1 ± 0.5	1.6 ± 0.8	1.3 ± 0.6	1.1 ± 0.6	1.3 ± 0.5	1.3 ± 0.6	0.148	0.237	
		B	0.8 ± 0.1	1.3 ± 0.7	1.7 ± 1.0	1.3 ± 0.5	1.6 ± 1.2	1.0 ± 0.4	0.104		
DNMT3a	EVOO	A	0.8 ± 0.3	0.9 ± 0.2	0.7 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	0.9 ± 0.4	0.198	0.569	0.539
		B	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.8 ± 0.3	0.9 ± 0.4	0.8 ± 0.3	0.375		
	ω3PUFA	A	1.1 ± 0.4	1.3 ± 0.6	1.0 ± 0.5	1.1 ± 0.3	1.2 ± 0.5	1.3 ± 0.7	0.336	0.356	
		B	1.0 ± 0.4	1.1 ± 0.4	1.0 ± 0.3	1.1 ± 0.4	0.9 ± 0.3	1.0 ± 0.1	0.777		
DNMT3b	EVOO	A	0.9 ± 0.5 a,b	0.7 ± 0.3 a	0.4 ± 0.2 a,b	0.5 ± 0.2 b	0.5 ± 0.2 b	0.6 ± 0.3 a,b	**0.021**	0.141	**0.020**
		B	0.7 ± 0.5	0.5 ± 0.2	0.5 ± 0.1	0.5 ± 0.3	0.6 ± 0.2	0.6 ± 0.4	0.494		
	ω3PUFA	A	1.1 ± 0.5	1.3 ± 0.5	1.0 ± 0.4	0.7 ± 0.3	0.8 ± 0.4	1.3 ± 0.6	**0.011**	**0.021**	
		B	1.2 ± 0.6 a,b	0.8 ± 0.2 a,b	1.0 ± 0.4 a	0.9 ± 0.3 a	0.7 ± 0.3 b	1.2 ± 0.6 a,b	**0.018**		
IL1B	EVOO	A	1.3 ± 1.0	1.7 ± 1.3	1.2 ± 1.1	1.7 ± 1.0	1.5 ± 0.8	1.5 ± 0.9	0.388	0.294	0.761
		B	1.1 ± 0.8	1.3 ± 0.9	1.3 ± 1.1	1.7 ± 1.0	1.5 ± 0.9	1.0 ± 0.4	**0.028**		
	ω3PUFA	A	1.1 ± 0.5	1.6 ± 0.7	1.4 ± 0.8	1.3 ± 0.6	1.6 ± 1.0	1.4 ± 0.5	0.321	0.411	
		B	1.0 ± 0.6 a	1.2 ± 0.8 a,b	1.6 ± 0.8 a,b	1.6 ± 0.5 b	1.6 ± 0.8 a,b	1.3 ± 0.6 a,b	0.079		
IL6	EVOO	A	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.6 ± 0.4	0.415	0.460	0.169
		B	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.3	0.6 ± 0.3	0.364		
	ω3PUFA	A	0.9 ± 0.4	0.8 ± 0.2	1.1 ± 0.6	0.7 ± 0.2	0.7 ± 0.2	1.6 ± 0.9	**0.030**	0.119	
		B	1.3 ± 0.8	0.7 ± 0.2	1.3 ± 0.5	0.8 ± 0.2	0.9 ± 0.6	1.0 ± 0.4	**0.048**		
PGC1a	EVOO	A	0.8 ± 0.5	1.4 ± 1.3	0.5 ± 0.5	0.8 ± 0.3	0.8 ± 0.5	0.9 ± 0.7	0.231	0.224	0.375
		B	0.8 ± 0.3	0.6 ± 0.1	0.7 ± 0.4	1.2 ± 1.3	1.0 ± 0.6	0.7 ± 0.6	0.338		
	ω3PUFA	A	1.5 ± 1.5	1.6 ± 1.1	1.1 ± 0.4	1.2 ± 0.9	1.2 ± 0.9	2.4 ± 2.4	0.195	0.085	
		B	0.8 ± 0.5	0.8 ± 0.7	0.6 ± 0.6	1.8 ± 1.7	1.3 ± 1.1	0.7 ± 0.4	0.159		
TNFα	EVOO	A	1.0 ± 0.6 a,b	1.2 ± 0.6 a,b	0.6 ± 0.4 a	0.8 ± 0.2 a,b	1.2 ± 0.4 b	1.2 ± 0.6 a,b	0.089	0.169	0.581
		B	1.0 ± 0.5	1.1 ± 0.5	0.7 ± 0.4	0.9 ± 0.2	0.8 ± 0.5	0.9 ± 0.7	0.447		
	ω3PUFA	A	1.1 ± 0.5	1.2 ± 0.2	0.9 ± 0.3	1.0 ± 0.2	1.1 ± 0.8	1.2 ± 0.5	0.401	0.341	
		B	1.0 ± 0.5	1.1 ± 0.4	1.3 ± 0.7	1.0 ± 0.2	0.9 ± 0.4	1.3 ± 0.6	0.296		

Gene expression of COX2, DNMT3a, DNMT3b, IL1B, interleukin-6 (IL6), PGC1α and tumor necrosis factor alpha (TNFα) cytokine levels pre and post eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as $2^{-(\Delta\Delta Ct)}$ fold change (relative to GAPDH expression and mean baseline value for each trial day) mean ± standard deviation. Means not sharing a letter (a,b)are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

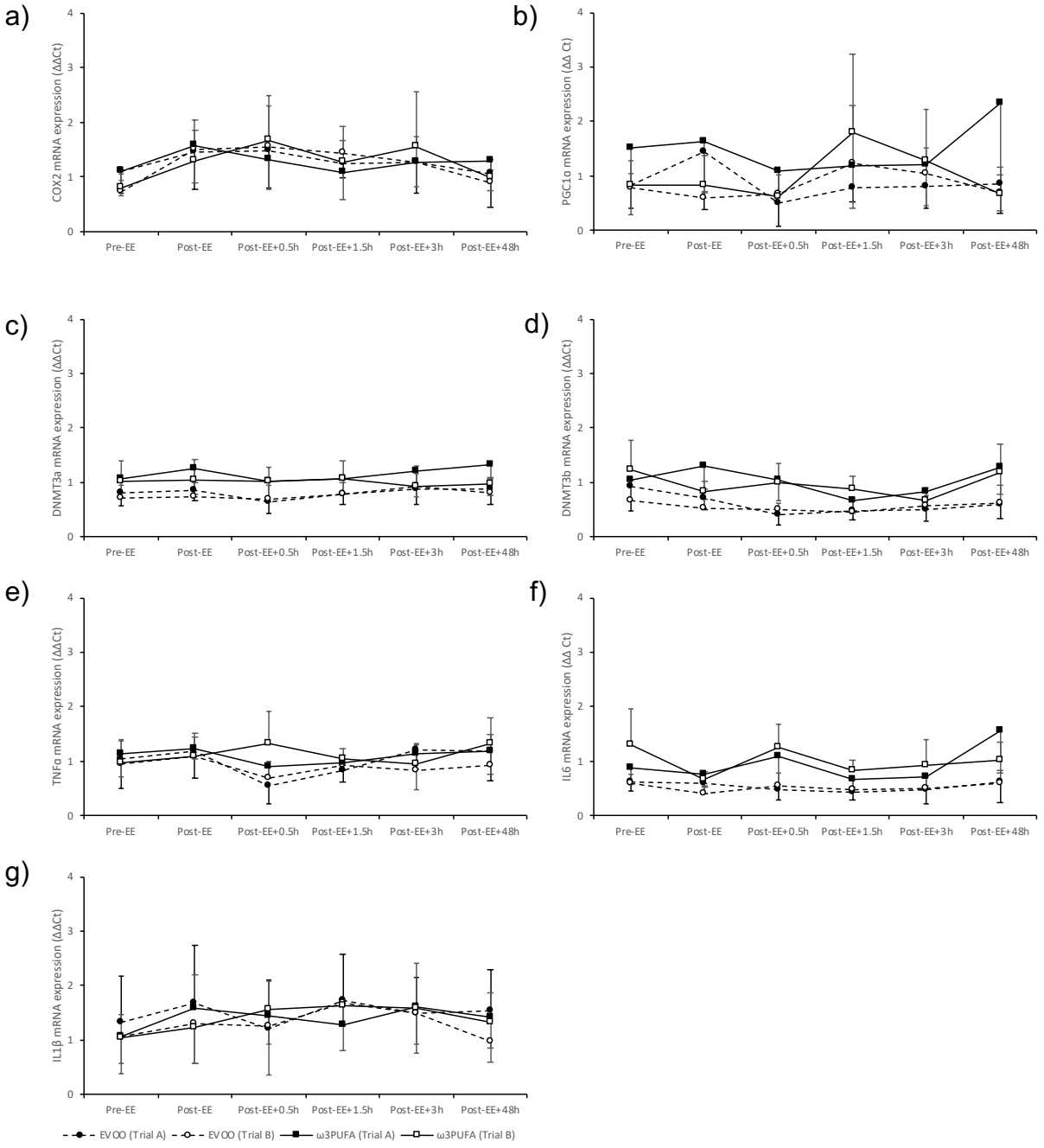


Figure 6-8 Gene expression of a) COX2, b) PGC1α, c) DNMT3a, d) DNMT3b, e) TNFα f) IL6, g) IL1β pre and post eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA). Data presented as $2^{-(\Delta\Delta Ct)}$ fold change (relative to GAPDH expression and mean baseline value for each trial day) mean ± 95% confidence intervals.

6.3.8 DNA methylation

We measured the percentage of methylated CpG sites of interest in three genes, *TNF*, *IL1B* and *IL6*.

6.3.8.1 *Tumor necrosis factor (TNF)*

The percentage methylation values of four CpG sites in *TNF* were measured and the results are presented together with the average methylation of the four sites in Table 6-11. Low methylation levels of *TNF* (<15%) were observed at all the CpG sites measured.

Analysis of the results for all participants during Trial A shown in Figure 6-9 indicate that the methylation of *TNF* decreased following EE and had returned to pre-exercise levels 48 hours later (CpGavg $F(3.254,48.803)=8.841$, $p<0.001$). In general, the same trend was seen across the measured time points at each site and for each trial regardless of the supplementation.

The results of the two way ANOVA (time x trial) indicated that there were no significant interactions in methylation of *TNF* between Trial A and Trial B and there were no significant three-way interactions between time, trial and supplement either, visualised for CpG2 in Figure 6-10.

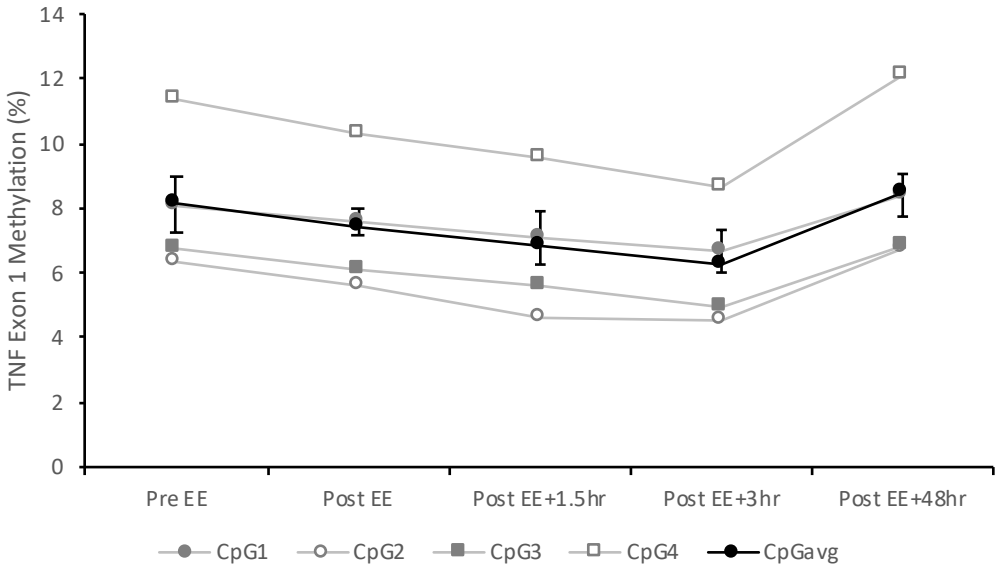


Figure 6-9 Average DNA methylation of four CpG sites (CpGavg) within Tumor necrosis factor (TNF) exon1 pre- and post-eccentric exercise (EE). Data presented as mean and ±95% confidence intervals for CpGavg.

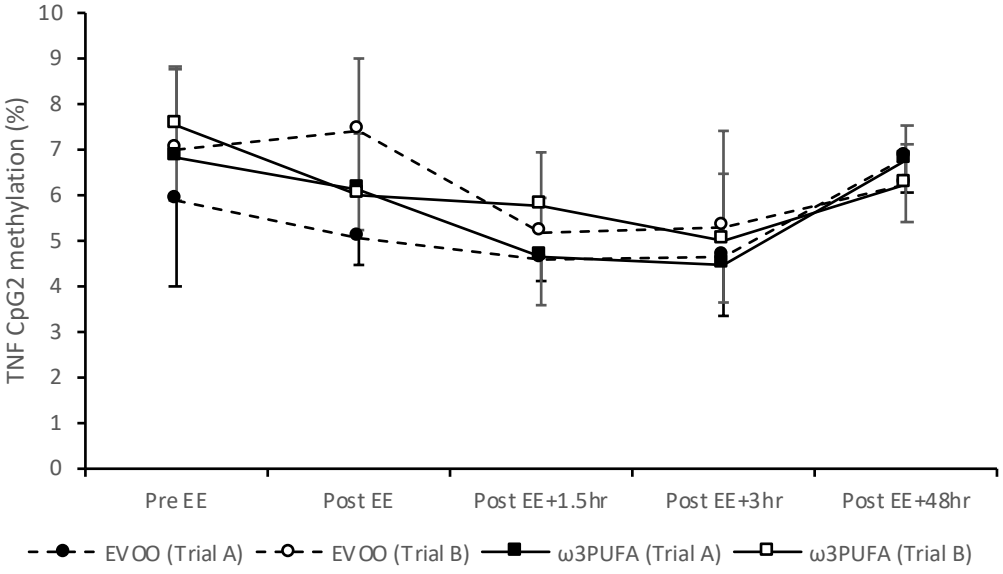


Figure 6-10 DNA methylation CpG2 within the Tumor necrosis factor (TNF) exon1 pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ±95% confidence intervals.

Table 6-11 Tumor Necrosis Factor (TNF) exon 1 methylation levels

CpG Site	Supplement	Trial	Pre-EE	Post-EE	Post-EE+1.5h	Post-EE+3h	Post-EE+48h	p simple interaction $	p two-way #	p three-way §
CpG1	EVOO	A	8.4 ± 1.6	7.4 ± 0.9	7.2 ± 1.2	7.1 ± 1.8	8.2 ± 1.1	0.176	0.202	0.212
		B	8.6 ± 1.2 a,b	10.0 ± 2.0 a	6.9 ± 1.2 b	7.7 ± 2.4 a,b	8.9 ± 1.3 a,b	**0.020**		
	ω3PUFA	A	7.9 ± 1.0	7.8 ± 1.0	7.0 ± 1.2	6.3 ± 1.3	8.7 ± 1.8	**0.034**	0.642	
		B	9.2 ± 1.1	8.1 ± 1.9	7.6 ± 1.7	7.5 ± 1.9	8.6 ± 0.6	0.145		
CpG2	EVOO	A	5.9 ± 1.9 a,b	5.1 ± 0.6 a	4.6 ± 0.5 a	4.6 ± 1.3 a	6.8 ± 0.8 a,b	**0.011**	0.099	0.176
		B	7.0 ± 1.8	7.4 ± 1.6	5.2 ± 0.8	5.3 ± 2.1	6.2 ± 1.3	**0.021**		
	ω3PUFA	A	6.8 ± 1.0 a,b	6.1 ± 0.9 a	4.6 ± 1.0 b,c	4.5 ± 0.8 c	6.7 ± 1.4 a,b,c	**0.001**	0.303	
		B	7.5 ± 1.3 a	6.0 ± 1.4 b	5.8 ± 1.2 a,b	5.0 ± 1.5 b	6.2 ± 0.9 a,b	**0.016**		
CpG3	EVOO	A	6.7 ± 2.3	6.0 ± 0.8	5.6 ± 1.4	5.2 ± 1.1	6.4 ± 1.3	0.214	0.470	0.549
		B	7.4 ± 1.0	7.8 ± 1.1	5.9 ± 1.1	5.8 ± 2.2	6.4 ± 0.9	**0.030**		
	ω3PUFA	A	6.9 ± 0.8 a	6.2 ± 1.1 a,b	5.7 ± 1.5 a,b	4.7 ± 0.6 b	7.3 ± 1.5 a,b	**0.007**	0.799	
		B	7.8 ± 0.8	6.4 ± 1.1	6.2 ± 1.7	5.7 ± 1.7	7.7 ± 0.8	**0.017**		
CpG4	EVOO	A	11.2 ± 3.1 a,b	10.2 ± 1.0 a,b	9.7 ± 2.2 a	8.8 ± 2.1 a	12.1 ± 1.6 b	**0.031**	0.156	0.539
		B	12.6 ± 1.8 a	12.4 ± 2.0 a	8.8 ± 1.6 b	9.8 ± 2.5 a,b	11.4 ± 1.7 b	**0.001**		
	ω3PUFA	A	11.5 ± 1.5 a	10.5 ± 0.9 a,b	9.4 ± 2.1 a,b	8.5 ± 1.2 b	12.1 ± 2.0 a,b	**0.004**	0.777	
		B	13.0 ± 2.1 a,b	11.6 ± 1.7 a,b	10.5 ± 2.2 a,b	9.5 ± 2.0 a	12.2 ± 1.3 b	**0.028**		
CpGavg	EVOO	A	8.1 ± 2.1 a,b	7.2 ± 0.6 a,b	6.8 ± 1.6 a	6.6 ± 1.5 a,b	8.3 ± 1.0 b	0.068	0.255	0.454
		B	8.9 ± 1.3 a,b	9.4 ± 1.6 a,b	6.8 ± 1.3 a	7.2 ± 2.2 a,b	8.2 ± 1.2 b	**0.014**		
	ω3PUFA	A	8.2 ± 1.0	7.7 ± 0.9	6.8 ± 1.6	6.0 ± 0.9	8.7 ± 1.6	**0.006**	0.734	
		B	9.4 ± 1.3	8.0 ± 1.4	7.5 ± 1.6	6.9 ± 1.7	8.7 ± 0.8	**0.024**		

DNA methylation of *TNF* pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± standard deviation. Means not sharing a letter (a,b,c) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

6.3.8.2 *Interleukin 1beta (IL1B)*

The percentage methylation of five CpG sites in *IL1B* were measured, the results are shown in Table 6-12. High methylation levels of *IL1B* (>70%) were observed, compared to *TNF.* The methylation level of CpG5 was less than that observed for the other *IL1B* sites.

Analysis of the results for all participants during Trial A shown in Figure 6-11 indicate that the methylation of *IL1B* was significantly altered after EE (CpGavg: $F(2.618,39.272)=8.418$, $p<0.001$. Methylation was seen to increase following the exercise and had returned to pre-exercise levels 48 hours later.

The changes in recorded methylation at CpG3 (Figure 6-12 A) were significant in both Trail A and Trial B for both supplementation groups, although no significant interactions were shown between pre- and post-supplementation results or supplementation type.

The results for CpG5 (Figure 6-12 B) of *IL1B* were interesting in that all measurements for this site showed that it was generally less methylated than the other sites measured for this gene. It also appeared that there were slight increases to the methylation levels following EE and that these returned to pre-exercise levels 48 hours later but the statistical significance of this effect was not clear. The results of the three-way ANOVA indicate that there was an interaction between the supplement and trials for each time point.

The EVOO supplemented group saw and increase in methylation at CpG5 as a result of EE before supplementation; however, after taking the EVOO supplement, there was no change in methylation levels following EE. In contrast, the ω-3 PUFA group did show changes to the level of methylation of CpG5 following EE both before (not significantly) and after supplementation. The interaction between Trial A and Trial B for the ω-3 PUFA group was significant ($p=0.013$), which is the result of the methylation level of the 48-hour measurement after supplementation being significantly lower ($p=0.027$) than the pre-exercise level. At CpG5 the difference in response to exercise between the supplement groups at Trial B results in a significant three-way interaction (time x trial x supplement, $F(2.893,40.498)=3.368$, $p=0.029$).

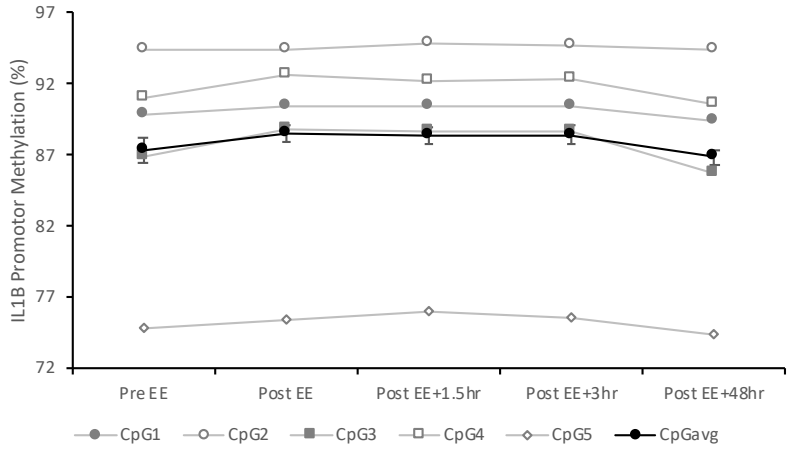
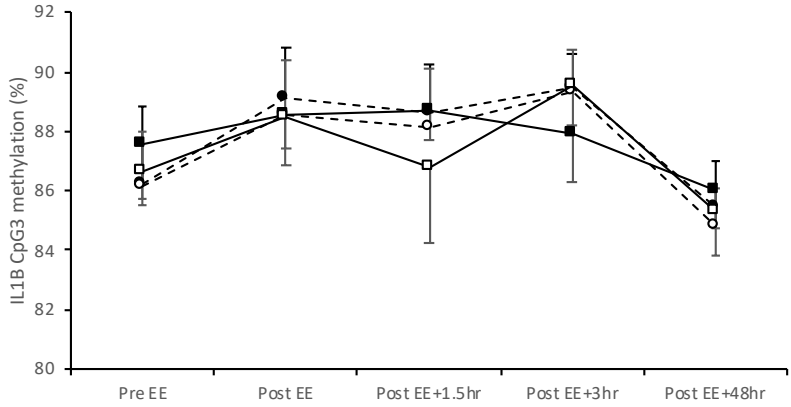


Figure 6-11 Average DNA methylation of five CpG sites (CpGavg) within Interleukin 1B (IL1B) promotor pre and post-eccentric exercise (EE). Data presented as mean and ±95% confidence intervals for CpGavg

A)



B)

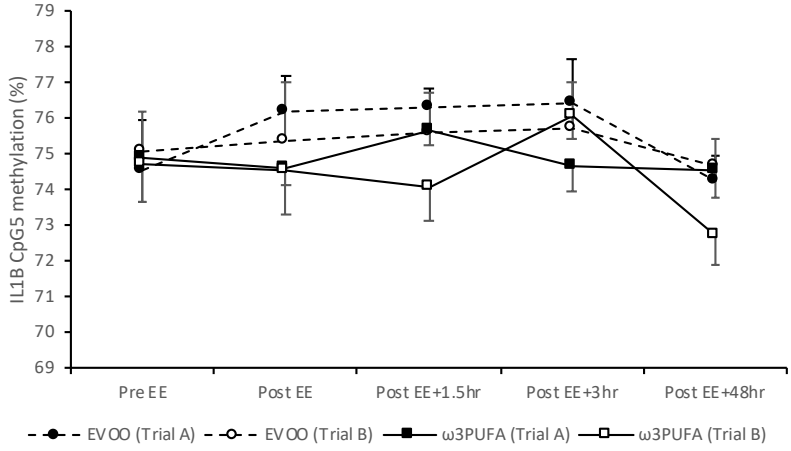


Figure 6-12 DNA methylation A) CpG2 B) CpG5 within the Interleukin 1beta (IL1B) promotor pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ±95% confidence intervals.

Table 6-12 Interleukin 1 beta (IL1B) methylation levels

CpG Site	Supplement	Trial	Pre-EE	Post-EE	Post-EE+1.5h	Post-EE+3h	Post-EE+48h	p simple interaction $	p two-way #	p three-way §
CpG1	EVOO	A	89.5 ± 2.7 a,b	91.1 ± 2.2 a,b	90.7 ± 2.4 a,b	91.8 ± 1.8 a	89.6 ± 1.7 b	**0.047**	0.614	0.559
		B	90.1 ± 3.2 a,b	90.9 ± 3.4 a,b	91.6 ± 2.7 a,b	92.1 ± 1.9 a	88.5 ± 2.4 b	0.097		
	ω3PUFA	A	90.2 ± 1.1	89.8 ± 1.4	90.1 ± 1.1	89.2 ± 0.5	89.2 ± 1.3	0.219	0.197	
		B	89.1 ± 2.2 a,b	89.7 ± 2.2 a,b	89.8 ± 2.8 a,b	90.6 ± 1.6 a	87.7 ± 1.6 b	0.077		
CpG2	EVOO	A	94.4 ± 0.8	94.6 ± 0.4	94.7 ± 0.9	94.8 ± 0.5	94.3 ± 0.7	0.485	0.495	0.945
		B	93.5 ± 0.9 a	94.4 ± 1.0 a,b	94.1 ± 0.8 a,b	95.0 ± 0.7 b	93.9 ± 0.8 a,b	**0.030**		
	ω3PUFA	A	94.3 ± 0.7	94.3 ± 0.4	94.8 ± 0.9	94.6 ± 0.8	94.4 ± 0.9	0.442	0.366	
		B	93.6 ± 1.2	94.4 ± 0.9	94.0 ± 0.7	94.6 ± 0.6	94.1 ± 0.5	0.237		
CpG3	EVOO	A	86.2 ± 3.1 a,b	89.1 ± 2.0 a	88.6 ± 2.0 a,b	89.4 ± 1.4 a	85.5 ± 1.8 b	**0.004**	0.983	0.523
		B	86.1 ± 2.2 a,c	88.6 ± 2.2 b,c	88.1 ± 2.4 a,b,c	89.3 ± 1.8 a,b	84.8 ± 1.5 c	**0.006**		
	ω3PUFA	A	87.5 ± 2.2 a,b	88.5 ± 1.4 a	88.7 ± 1.2 a,b	87.9 ± 1.9 a,b	86.0 ± 1.5 b	**0.031**	0.187	
		B	86.6 ± 1.3 a,b	88.5 ± 2.0 a,b	86.8 ± 3.0 a,b	89.6 ± 1.6 a	85.3 ± 1.7 b	**0.012**		
CpG4	EVOO	A	90.4 ± 2.3	93.0 ± 1.5	91.7 ± 2.3	92.6 ± 1.5	90.6 ± 2.1	**0.012**	0.610	0.378
		B	90.1 ± 2.4 a,b	91.8 ± 2.0 a,b	92.2 ± 2.6 a,b	92.6 ± 1.9 a	89.7 ± 1.9 b	**0.027**		
	ω3PUFA	A	91.6 ± 1.9 a,b	92.2 ± 1.2 a,b	92.6 ± 1.5 a	91.9 ± 1.6 a,b	90.6 ± 1.1 b	0.125	0.365	
		B	91.2 ± 1.4 a,b	92.2 ± 1.5 a	90.8 ± 3.0 a,b	92.2 ± 1.4 a	89.3 ± 1.4 b	0.066		
CpG5	EVOO	A	74.6 ± 1.7 a,b	76.2 ± 1.2 a,b	76.3 ± 0.6 a	76.4 ± 1.4 a,b	74.2 ± 0.8 b	**0.003**	0.484	**0.029**
		B	75.1 ± 1.3	75.4 ± 1.9	75.6 ± 1.3	75.7 ± 1.6	74.6 ± 0.9	0.530		
	ω3PUFA	A	74.9 ± 1.5 a,b	74.6 ± 0.5 a,b	75.7 ± 0.5 a	74.7 ± 0.8 b	74.5 ± 0.9 a,b	0.165	**0.013**	
		B	74.7 ± 1.2 a,b	74.5 ± 1.5 a,b,c	74.1 ± 1.1 a,c	76.1 ± 0.8 b	72.7 ± 1.0 c	**0.002**		
CpGavg	EVOO	A	87.0 ± 2.0 a,b	88.9 ± 1.2 a	88.4 ± 1.4 a	89.1 ± 1.1 a	86.7 ± 1.0 b	**0.003**	0.936	0.331
		B	87.0 ± 1.6 a,c	88.3 ± 1.8 b,c	88.3 ± 1.9 a,b,c	88.9 ± 1.1 a,b	86.3 ± 1.3 c	**0.036**		
	ω3PUFA	A	87.7 ± 1.3 a,b	88.0 ± 0.8 a	88.4 ± 0.8 a,b	87.7 ± 0.9 a,b	86.9 ± 0.8 b	0.056	0.111	
		B	87.1 ± 0.9 a,b	87.8 ± 1.3 a,b	86.9 ± 2.0 a,b	88.6 ± 0.9 a	85.9 ± 1.0 b	**0.015**		

DNA methylation of *IL1B* pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± standard deviation. Means not sharing a letter (a,b,c) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

6.3.8.3 *Interleukin 6 (IL6)*

The percentage methylation of six CpG sites in *IL6* were measured, the results are shown in Table 6-13. As with *IL1B*, the levels of methylation at all the CpG sites within the region of *IL6* that were studied were high (>80%).

The combined results for methylation of *IL6* promotor during Trial A, shown in Figure 6-13, showed no significant response to EE, with the exception of CpG1 and CpG6. The significant change in CpG6 methylation over the time points measured $F(3.289,49.276)=3.832$, $p=0.013$ remained significant after correction for multiple testing with a decrease (89.7% vs. 88.3%, $p=0.007$) of 1.4% at 3 hours post EE. The change in methylation at CpG1 was not significant after correction for multiple testing.

When comparing resting methylation levels before and after supplementation, *IL6* CpG1 methylation had increased in the ω-3 PUFA group (90.5 ± 1.3 vs. 91.2 ± 1.2) compared to a decrease in the EVOO group (91.5 ± 2.3 vs. 90.6 ± 2.0), with the interaction almost reaching statistical significance ($p=0.055$).

After supplementation, other than at CpG2 for the EVOO supplemented group, no significant changes were seen to methylation levels of any of the CpG sites measured in *IL6*. After supplementation with EVOO, the methylation of CpG2 was significantly higher three hours after EE compared to Trial B Pre-EE values (90.7% vs. 92.4%, $p=0.015$).

There were no significant two-way (time x trial) or three-way (time x trial x supplement) interactions observed for *IL6* methylation.

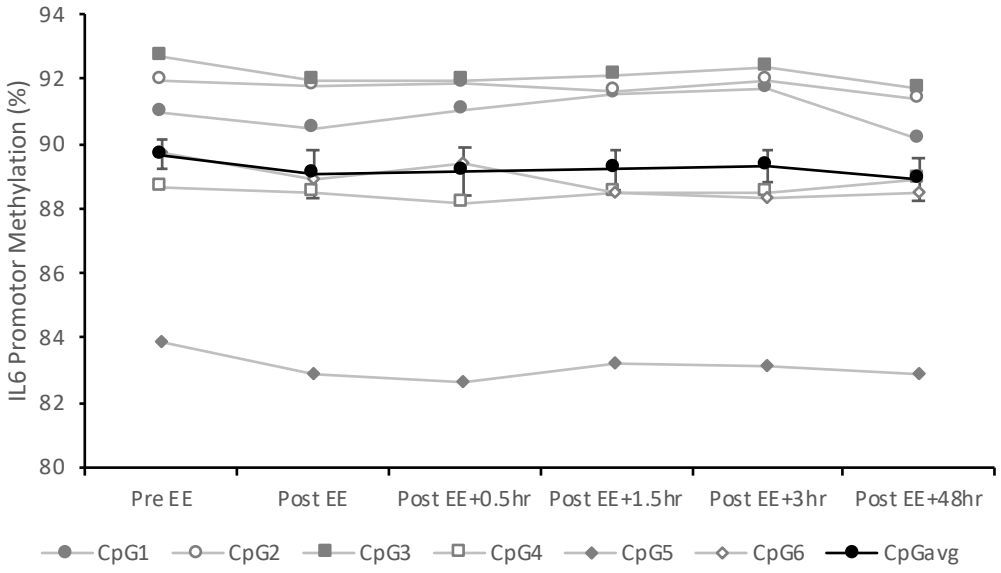


Figure 6-13 Average DNA methylation of six CpG sites (CpGavg) within Interleukin 6 (IL6) promotor pre- and post-eccentric exercise (EE). Data presented as mean and ±95% confidence intervals for CpGavg during Trial A (n=16).

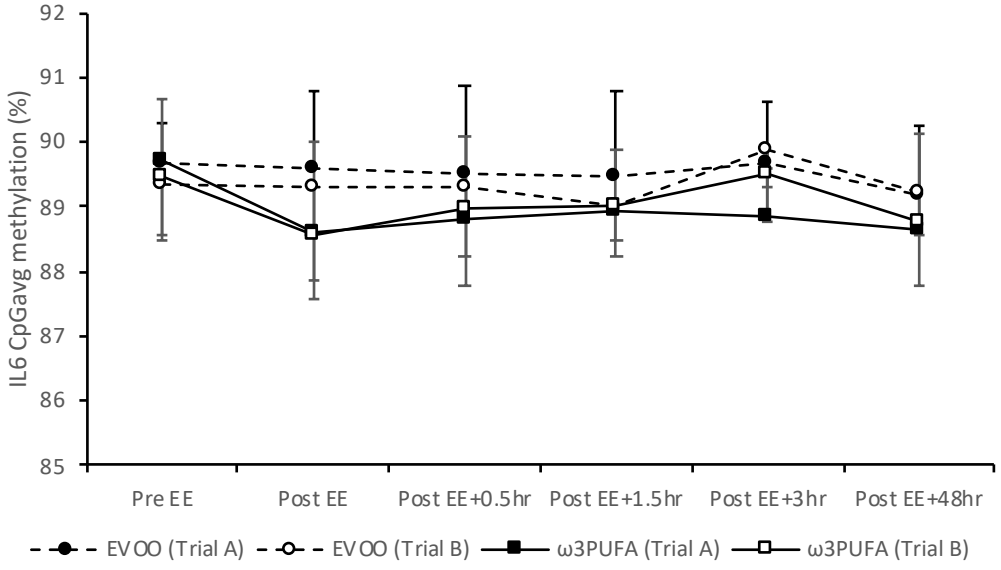


Figure 6-14 DNA methylation CpG average (CpGavg) within the Interleukin 6 (IL6) promotor pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ±95% confidence intervals

Table 6-13 Interleukin 6 (IL6) promotor methylation levels

CpG Site	Supplement	Trial	Pre-EE	Post-EE	Post-EE+0.5h	Post-EE+1.5h	Post-EE+3h	Post-EE+48h	p simple interaction $	p two-way #	p three-way §
CpG1	EVOO	A	91.5 ± 2.3	90.9 ± 2.0	91.5 ± 2.7	92.0 ± 2.4	92.3 ± 2.6	89.9 ± 2.2	0.072	0.534	0.496
		B	90.6 ± 2.0	90.8 ± 2.5	91.2 ± 1.8	91.3 ± 0.9	91.6 ± 1.4	90.7 ± 2.1	0.532		
	ω3PUFA	A	90.5 ± 1.3	90.0 ± 1.0	90.6 ± 1.5	91.1 ± 1.4	91.1 ± 1.1	90.4 ± 1.3	0.368	0.638	
		B	91.2 ± 1.2	90.1 ± 1.7	90.9 ± 1.7	90.5 ± 0.8	91.7 ± 1.0	90.4 ± 1.4	0.243		
CpG2	EVOO	A	91.8 ± 0.8	92.1 ± 1.1	92.1 ± 1.0	91.7 ± 1.5	92.2 ± 0.7	91.4 ± 1.0	0.411	0.455	0.948
		B	90.7 ± 1.2 a	91.8 ± 1.2 a,b	91.6 ± 0.8 a,b	91.6 ± 0.5 a,b	92.4 ± 0.6 b	90.9 ± 1.1 a,b	**0.018**		
	ω3PUFA	A	92.1 ± 1.6	91.5 ± 1.0	91.7 ± 0.7	91.6 ± 1.3	91.6 ± 0.8	91.4 ± 1.0	0.565	0.263	
		B	91.0 ± 1.2	91.5 ± 1.6	91.6 ± 1.2	91.5 ± 0.6	92.4 ± 0.8	91.5 ± 1.0	0.269		
CpG3	EVOO	A	92.3 ± 0.8	92.7 ± 1.8	92.3 ± 1.8	92.3 ± 2.2	92.6 ± 1.6	92.2 ± 1.4	0.822	0.478	0.850
		B	92.0 ± 1.2 a,b	92.3 ± 1.8 a,b	91.9 ± 1.8 a,b	91.3 ± 1.6 a	92.7 ± 1.4 b	91.8 ± 1.0 a,b	0.242		
	ω3PUFA	A	93.0 ± 1.6	91.2 ± 2.6	91.7 ± 2.1	92.0 ± 1.8	92.2 ± 0.4	91.3 ± 2.3	0.254	0.834	
		B	92.5 ± 1.4	91.5 ± 1.8	92.0 ± 2.7	91.8 ± 2.1	92.3 ± 2.2	91.9 ± 1.3	0.760		
CpG4	EVOO	A	88.8 ± 0.7	87.8 ± 1.9	88.1 ± 0.9	88.3 ± 1.4	88.2 ± 1.0	88.6 ± 0.7	0.541	0.791	0.526
		B	88.5 ± 1.5	87.6 ± 1.5	88.4 ± 1.4	88.3 ± 0.9	88.9 ± 0.9	88.4 ± 1.2	0.434		
	ω3PUFA	A	88.6 ± 1.2	89.2 ± 1.1	88.2 ± 0.8	88.8 ± 0.8	88.7 ± 0.9	89.2 ± 1.7	0.255	0.112	
		B	89.6 ± 2.3	88.1 ± 1.9	88.5 ± 1.7	88.9 ± 0.9	89.7 ± 1.5	88.4 ± 1.2	0.174		
CpG5	EVOO	A	83.9 ± 0.8	84.2 ± 1.8	83.2 ± 2.6	83.8 ± 1.3	84.1 ± 1.4	83.8 ± 2.0	0.670	0.416	0.846
		B	84.0 ± 1.5	83.0 ± 2.2	83.6 ± 1.9	82.8 ± 0.9	84.1 ± 1.8	83.5 ± 1.0	0.414		
	ω3PUFA	A	83.8 ± 1.7	81.7 ± 3.3	82.1 ± 2.5	82.8 ± 1.6	82.2 ± 2.7	81.9 ± 4.5	0.248	0.718	
		B	83.5 ± 1.2	81.5 ± 1.7	82.7 ± 2.3	82.5 ± 1.9	82.7 ± 1.9	82.4 ± 2.8	0.153		
CpG6	EVOO	A	89.6 ± 1.3	89.8 ± 1.6	89.9 ± 2.4	89.0 ± 1.4	88.8 ± 1.0	89.3 ± 1.6	0.323	0.332	0.346
		B	90.3 ± 1.5 a,b	90.3 ± 1.4 a	89.1 ± 1.4 a,b	88.8 ± 0.9 b	89.7 ± 1.4 a,b	89.6 ± 0.5 a,b	0.123		
	ω3PUFA	A	89.9 ± 0.8 a	88.0 ± 1.9 a,b	88.9 ± 1.9 a,b	88.1 ± 1.5 a,b	87.8 ± 1.3 b	87.8 ± 2.5 a,b	**0.031**	0.138	
		B	88.9 ± 2.0	89.0 ± 1.0	88.2 ± 1.4	88.3 ± 0.8	88.3 ± 1.2	88.5 ± 0.7	0.418		
CpGavg	EVOO	A	89.7 ± 0.6	89.6 ± 1.2	89.5 ± 1.4	89.5 ± 1.3	89.7 ± 0.9	89.2 ± 1.0	0.635	0.663	0.960
		B	89.3 ± 0.9 a,b	89.3 ± 1.4 a,b	89.3 ± 1.0 a,b	89.0 ± 0.5 a	89.9 ± 0.6 b	89.2 ± 0.7 a,b	0.326		
	ω3PUFA	A	89.7 ± 1.0	88.6 ± 1.4	88.8 ± 1.3	89.0 ± 0.9	88.9 ± 0.7	88.7 ± 1.5	0.181	0.634	
		B	89.5 ± 0.9	88.6 ± 1.0	89.0 ± 1.2	89.0 ± 0.8	89.5 ± 0.7	88.8 ± 1.0	0.199		

DNA methylation of *IL6* pre- and post-eccentric exercise (EE) completed before (Trial A) and after (Trial B) 21 days of either extra virgin olive oil (EVOO) or omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplementation. Data presented as mean ± standard deviation. Means not sharing a letter (a,b,c) are significantly different for simple interactions of time ($p<0.05$) after Bonferroni correction for multiple testing. § Three-way mixed ANOVA (time x trial x supplement). # Two-way repeated measures ANOVA (time x trial). $ One-way repeated measures ANOVA (time, within trial).

6.3.9 Associations between DNA methylation and gene expression

The associations between the methylation status of the CpG sites that were studied and other variables measured (anthropometric measurements, gene expression, proteins and methylation of the other sites) before supplementation is depicted in the form of a 'heat map' in Figure 6-15.

Negative associations are shown in red and positive associations in green, with the shade of colour representing the strength of the association. A large number of associations are shown between individual sites and these are not commented upon in detail, however attention is drawn to the obvious apparent associations below.

Baseline associations, which depict the position before muscle damage, are displayed in the first three columns. There is a negative association between the studied methylation status of each of the four CpG sites in *TNF* and five of the six CpG sites in *IL1B*. The exception being that CpG2 of *IL1B* showed no association with any of the *TNF* sites. Immediately after the muscle damaging exercise the negative association described above between *TNF* and *IL1B* had substantially disappeared and both at three and 48 hours after the completion of the exercise the initial association remained absent.

In contrast to the association described above between *TNF* and *IL1B*, very little association was observed between the methylation status of *IL6* and *IL1B* at baseline but immediately after the muscle damaging exercise a positive association was observed between four of the *IL6* sites and five of the *IL1B* sites and a new negative association was observed between two sites in *TNF* and three in *IL6*. At three hours after the exercise the positive association between the methylation status of the *IL6* and *IL1B* sites appeared to be less but this was slightly stronger after 48 hours.

Methylation of the *TNF* gene was negatively associated with TNFα mRNA levels.

As regards the anthropometric measurements negative association between body fat percentage and *TNF* methylation of three of the studied CpG sites were observed before muscle damage and supplementation, but this was not observed at any point thereafter. The methylation of CpG2 was also negatively associated with BMI and positively associated with water weight.

Significant ($p<0.05$) Spearman's correlation coefficients. Methylation of cytosine-guanine dinucleotide (CpG) sites for IL6, IL1B and TNF; average methylation of the four sites (CpGavg) Before (Pre), and after (Post-EE, Post-EE+3h and Post-EE+48h) Eccentric Exercise (EE). Red indicates negative correlation, green indicates positive correclation and black indicates no significant correlation, see key.

Figure 6-15 Correlations between blood DNA methylation of Interleukin 6 (IL6), Interleukin 1beta (IL1B) and Tumor necrosis factor (TNF) with each other , gene expression and serum protein levels. Data values in Appendix 4.

6.4 Discussion

The aim of the research presented in this chapter was to investigate whether the methylation profiles of three cytokine genes would change in the presence of an inflammatory stimulus. The three genes studied were *TNF*, *IL6* and *IL1B,* and an intense bout of exercise was used to provide the inflammatory stimulus. This research also sought to investigate if the observed results would be different dependent upon whether the cohort was supplemented with either ω-3 PUFAs or EVOO.

It has previously been established that individuals who are unaccustomed to EE suffer varying degrees of muscle damage as a result of the eccentric contractions and that a similar response is not observed as a result of concentric contractions (Brown, Day and Donnelly, 1999). Therefore, the chosen inflammatory stimulus was EE. To establish whether EE had successfully provided this stimulus, the participant's perception of pain, their range of movement around affected joints and muscle swelling were measured. All of this information was recorded at baseline and after EE and then again after the supplementation intervention and a second bout of the exercise. Eccentric exercise results in the release of CK, LDH and Mb when muscle fibres are damaged leading to increased levels within serum (Brown, Day and Donnelly, 1999). Therefore, in addition to the perceived pain and physical measurements, biochemical measurements of these three muscle damage markers from the participant's blood in order to establish whether muscle damage had occurred.

The data collected regarding pain perception confirmed that the participants felt increased muscle discomfort following the EE and that this discomfort was still apparent after 48 hours. The ROM around the affected joints decreased indicating that flexibility within the muscles was reduced after EE, but measurements of thigh girth were unable to detect signs of swelling. As regards the measurement of muscle damage markers in the blood, it was found that levels of CK and Mb increased after EE and that they remained elevated 48 hours after the exercise had been performed. However, the expected increase in LDH levels were not observed, only a trend can be seen towards elevated levels, but these were not statistically significant. Nevertheless, taken together, all of these results do indicate that the EE undertaken

had achieved the desired effect of damaging muscle fibres and thus stimulating an inflammatory response.

The intention of providing the inflammatory stimulus was to see whether it would alter the methylation levels of three genes which encode proteins known to be involved in inflammation. Eccentric exercise was not found to have any effect on the levels of methylation within the *IL6* promotor region, with the exception of a small decrease at CpG6, however it was established that EE induced demethylation of four CpG sites within the first exon of the *TNF* gene and that it induced increased methylation at three sites in the promotor of *IL1B*.

In response to EE, a small but significant decrease in the methylation of a single CpG (CpG6, -1057) *in IL6* was observed, but no significant changes were detected at any of the other sites measured. The observed decrease corresponded with an increase in serum levels of the IL6 cytokine after EE, however whilst cytokine levels returned towards baseline levels by the 48-hour time point, methylation levels had not. Previous research investigating the methylation of *IL6* has detailed the importance of methylation upstream of the promotor in the control of *IL6* expression (Poplutz *et al.*, 2014). When PBMCs were stimulated with LPS, the methylation of the CpG -1099bp from the TSS was found to be significantly lower in those with increased mRNA (Nile *et al.*, 2008). Here there was no change in IL6 mRNA observed. The control of *IL6* expression through DNA methylation does not just involve these CpG sites where, as observed here and in other research, the methylation levels are very high (Na *et al.*, 2015). The findings here, which are in agreement with the literature (Nile *et al.*, 2008; Na *et al.*, 2015), indicate that decreased methylation of sites 1000bp from the TSS are involved with the epigenetic control of IL6 cytokine levels. Further research, not restricted to the number of sites that can be measured, would provide additional understanding of the effects of exercise on IL6 expression through other CpG sites which have been shown to control expression in health and disease (Kirchner *et al.*, 2014; Poplutz *et al.*, 2014; Ma *et al.*, 2016).

The decrease in methylation seen in the *TNF* gene was much clearer than that observed in *IL6*, where it was observed at all four CpG sites measured. The lowest levels of methylation at *these* sites were measured three hours after EE; with levels having returned to baseline values 48 hours later. Serum TNFα has previously been

shown to negatively correlate with methylation levels in the promotor (Zhang *et al.*, 2017) and exon 1 (Hermsdorff *et al.*, 2013) of the *TNF* gene. Therefore, the demethylation of *TNF* which was observed would be expected to increase the gene expression and lead to increased serum levels of TNFα. Unexpectedly, a decrease in gene expression was observed even though there was no expected increase in TNFα levels. Whilst it is possible that the expression of *TNF* could have been transient, and that the time points where an increase had occurred were not measured, the regulation of TNFα cytokine production during an inflammatory response is known to be a tightly regulated process in which both transcriptional and post-transcriptional mechanisms are involved (Aslam and Zaheer, 2011). Hence it is possible that the observed reduction in *TNF* mRNA was the result of degradation by factors such as adenylate-uridylate-rich element and zinc finger protein 36 (Dean *et al.*, 2003). Whilst this may explain why this research failed to observe the expected results, it is also possible that issues in the collection of RNA from blood samples, as discussed in detail in Section 7.3.2, were the cause.

In contrast to the decreased methylation of *TNF*, three CpG sites within the promotor of *IL1B* displayed increased levels of methylation after EE. Methylation at these sites had also returned to baseline levels 48 hours after exercise had ceased. These observed methylation changes were not accompanied by any change in expression levels of IL1B transcript, and unfortunately due to experimental issues with the bead array kit, levels of serum IL1β could not be detected. Previous research into the effects of resistance exercise on the expression of *IL1B* from chondrocytes in females with and without rheumatoid arthritis has found decreased levels of IL1β cytokine after the exercise (Pereira Nunes Pinto *et al.*, 2017). In relation to the observed increase in methylation, theoretically this would reduce the expression of IL1B and therefore it would have expected to see a decrease in IL1β serum levels; as there was a lack of detection at baseline with the bead array kits, the reduction may have been even further out of the kits range of detection. DNA methylations proximal to the TSS in of *IL1B* have also been shown to have transcriptional control of the gene's expression (Hashimoto *et al.*, 2009, 2013). DNA methylation of the *IL1B* promotor may be one of the ways in which this response is controlled however, this research was unable to confirm this.

In order to understand how the changes in DNA methylation observed could potentially be controlled, the measurement of DNMT3a and DNMT3b gene expression was included. Interestingly, there was a decrease in the expression of DNMT3b as a result of the EE. In contrast, no changes in the levels of DNMT3a were observed. Previous research into the effects of exercise on the levels of DNMT enzymes has found that exercise reduces expression (Laye and Pedersen, 2010; Horsburgh *et al.*, 2015). These DNMT enzymes are responsible for de novo methylation of the genome. A decrease in the DNMT3b enzyme is in accordance with the decrease in methylation of *TNF* and *IL6*, however it does not account for the small increase in methylation of *IL1B*. Future investigation into the activity of the enzymes, rather than just the expression, would elucidate the mechanisms behind the observed changes in methylation. In addition, the measurement of enzymes involved in active removal of methyl groups should be included to understand how the decreases in methylation occur as a result of exercise (Wu and Zhang, 2014).

As described above, the decrease in levels of *TNF* methylation and the increase in levels of *IL1B* methylation which was observed to take place after EE had returned to base line 48 hours after the end of the exercise. To the authors knowledge, this study is the first to document these acute reversible changes to the methylation levels of these genes as a result of EE. The decrease in methylation of *IL6* at CpG6 which were observed in response to EE is also not documented elsewhere. Having established that the EE induced a change in the methylation levels of the candidate genes, this research was able to investigate whether supplementation with fatty acids interacted with these changes in any way.

Methylation levels for CpG1 (-1099) of *IL6* increased following ω-3 PUFA supplementation and decreased following EVOO supplementation, although the interaction did not reach significance ($p=0.05$). Methylation of a separate CpG closer to the TSS within the *IL6* genes has previously been associated with ω-3 PUFA in the blood (Ma *et al.*, 2016). Having regard to the aforementioned studies, and the changes seen in this research, the methylation of this particular site appears to be closely implicated in the expression of this gene and that expression appears to be influenced by levels of ω-3 PUFAs in the blood. There were no observed interactions at the CpG sites of *IL6* between the supplements and methylation levels after exercise. These

observations were similar as regards *TNF*, where the decrease in methylation observed when analysing all participants together remained once split by supplement group and after supplementation. The inability to detect changes in the methylation as a result of the fatty acids supplementations is likely to be affected by the small group sizes once split by supplement.

Within the *IL1B* gene, the methylation of CpG5 (-1009) was lower 1.5 hours and 48 hours after EE in the ω-3 PUFA group compared to EVOO group. In addition, the IL1B expression levels were increased 1.5 hours after the EE. This is in contrast to previous research which has observed decreases in IL1β as a result of ω-3 PUFA supplementation (Dangardt *et al.*, 2010). This, therefore, warrants future investigation to gain a better understanding of the interaction between the ω-3 PUFAs and *IL1B* methylation.

At Trial B, the participants were no longer unaccustomed to the EE as they had performed an intense bout during Trial A three weeks earlier. As expected, due to the repeated bout effect, although there was still an increase in CK and LDH during Trial B this was not of the same magnitude as that measured during Trial A. The repeated bout effect has been observed in individuals repeating the exercise at least six months apart (Nosaka *et al.*, 2001). Having regard to the repeated bout effect it is hard to delineate if the decrease in cytokine levels (IL6 and TNFα) which were observed in both groups after supplementation were as a result of the repeated exercise or of the supplementation.

Splitting the cohort into supplementation groups permitted comparison between those groups according to type of fatty acid. Previous supplementation research has found less elevation in the levels of Mb, LDH and CK in those supplemented with ω-3 PUFA compared to a placebo (SFA) and a control group (Tartibian, Maleki and Abbasi, 2011). No difference in Mb, CK and LDH release into the serum between the two supplements was observed in the current study. In comparison to the research design used here, where the participants complete two main ETDs, Tartibian *et al*. (2011) only required the participants perform the EE once at the end of the intervention and therefore as the participants were unaccustomed to the EE no repeated bout effect was observed.

The relationships between the methylation levels of the candidate genes and other measurements were assessed at Trial A by combining both supplement groups. Methylation levels of *TNF* exon 1 were negatively associated with the body fat of the participants at the start of the study. Previous research in a separate cohort of normal-weight young females, found that women with lower truncal fat had higher levels of methylation in the same CpG sites (Hermsdorff *et al.*, 2013). In addition, these CpG sites within the *TNF* gene have been used to predict response to weight loss intervention (Campión *et al.*, 2009). Furthermore, it was found that methylation of the *TNF* gene was negatively associated with *TNF* mRNA transcripts, a result which has previously been reported (Hermsdorff *et al.*, 2013; Marques-Rocha *et al.*, 2016).

Interestingly, there was a strong negative association between all *TNF* CpG sites measured and four of the *IL1B* CpG sites at baseline. This association did not remain after exercise, when *IL1B* methylation then switched to a positive association with *IL6* methylation. The pathways for each of these cytokines are complex and in fine balance. It is possible that these associations observed are a manifestation of this regulatory network.

6.4.1 Conclusion

In conclusion, it was found that methylation levels of specific sites within three inflammatory genes are altered by an intense bout of eccentric exercise with no effect of supplementation with either ω-3 PUFA or EVOO fatty acids.

Chapter 7 General Discussion

7.1 Summary of the research

The overall aim of this research was to increase knowledge and understanding of interactions between fatty acids and certain genes known to be associated with inflammation through the study of DNA methylation, an important epigenetic marker. This research also aimed to investigate whether it is possible to alter the methylation profile of selected inflammatory cytokine genes in order to promote a pro-resolution environment for inflammation.

In Chapter 1 the modern interpretation of the term epigenetics was explained and how expression of DNA can change in response to the environment. DNA methylation was introduced as one way in which the underlying DNA can remain unaltered whilst gene expression itself is changed and attention was drawn to evidence linking epigenetic changes to many diseases which are themselves associated with inflammation. Diet was identified as an important environmental factor and it was noted that there are popular dietary supplements, such as fish oil (ω-3 fatty acids), that could have epigenetic effects on inflammation.

The nature and importance of fatty acids was considered in Chapter 2, where it was also discussed what is meant by methylation. It was noted that methylation changes are a normal part of cell processes but that they can also be associated with disease and inflammation, which were considered in more detail. Current research into the epigenetic effects of fatty acids was reviewed with particular reference to the different ways in which methylation has been associated with fatty acid intake on a genome wide and candidate gene level. The research aims were then set out, which in the broadest sense were to study methylation of specific regions of genes known to be associated with inflammation and to see whether their methylation and expression would be altered in response to changes in the ω-3 fatty acid environment.

The experimental methodology for measuring the chosen epigenetic marker, DNA methylation, was detailed in Chapter 3 where it was discussed how methylation would be measured using bisulphite conversion and pyrosequencing. Assays were designed

and tested to enable measurement of methylation at biologically relevant sites in the chosen target genes and theses were used as the primary investigative tool.

The first piece of experimental research, which was reported and discussed in Chapter 4, was a cross sectional analysis of *TNF* methylation and associations with levels of fatty acids in the blood. This research particularly focused on whether methylation at exon-1 of this gene would be affected by the amount of fatty acids within the blood with a particular interest in ω-3 fatty acids. It was found that there are indeed significant relationships between levels of specific blood fatty acids and DNA methylation at the four CpG sites studied. Although methylation levels were not shown to be associated with most of the fatty acids measured, dependent upon the fatty acid group, some positive and some negative associations were observed, and it was also found that the amplitude and direction of associations differed according to sex.

These relationships were investigated further in Chapter 5 where it was considered whether changes to methylation could be provoked by supplementation altering the fatty acids profile of the blood. Having regard to the results of the previous study where it was found that more relationships between fatty acids and the methylation in males than females, and that the levels of ω-3 PUFA DHA were associated with *TNF* methylation, this second study had a cohort limited to males. The scope of the research was broadened by considering not just the *TNF* gene but two other genes, *IL6* and *IL1B*, both of which are also known to be associated with inflammation. However, this research was unable to detect any significant change to methylation over the four-week supplementation period and it was speculated that this might be because the healthy disease-free cohort had no inflammatory stimulus.

How an inflammatory stimulus might be provoked was considered at the beginning of Chapter 6. This final study set out to establish whether muscle damaging exercise would provoke a change in methylation levels of the candidate genes and whether there would be an increase in systemic inflammation. The results of various measurements of muscle damage and cytokine levels indicated that an inflammatory stimulus had been provided. Levels of methylation after the exercise were increased for *IL1B* and decreased for *IL6* and *TNF*. No effect of the supplementation was observed on the methylation of *IL1B* and *TNF*, whilst a small increase in *IL6* methylation was observed after exercise. Interactions between methylation levels after

exercise and supplementation were hard to detect and should be considered in future research.

To summarise, the following has been achieved in this thesis:

1. Designed and implemented robust assays for the measurement of DNA methylation of genes involved in inflammation (**Chapter 3**).
2. Determined that there was a relationship between DNA methylation of cytokine gene *TNF* and certain fatty acids found within the blood, with a difference in the relationship between males and females (**Chapter 4**).
3. Investigated the effect of a 28-day ω-3 PUFA supplement on the DNA methylation of cytokine the genes *TNF*, *IL6* and *IL1B*, finding no changes across the intervention (**Chapter 5**).
4. Profiled the DNA methylation levels of the cytokine genes *TNF*, *IL6* and *IL1B* before and after an intense bout of muscle damage inducing eccentric exercise, with an investigation into the interaction of fatty acid supplementation (**Chapter 6**).

7.2 Biological significance of findings

If this research is to be meaningful, understanding the broader biological significance of the findings is of great importance. The methylation of just 15 CpG sites within three genes was measured, out of the 28.3 million CpG sites in the human genome (Luo, Lu and Xie, 2014). A full understanding of the biological significance of these sites requires consideration of their biological relevance in the context of the small but statistically significant results that were observed and must be supported by phenotypic data such as gene expression and protein profiles. Although there were associations found between fatty acids and gene methylation as well as associations between methylation and gene expression, this research lacked mechanistic studies that could have delineated the direct effect.

The 15 CpG sites measured are a very small part of a larger and complex epigenetic network of mechanisms that fine tune the inflammatory response. Therefore, it is postulated that it is when the subtleties and fine tuning of this network fail that disruptions occur to the acute inflammatory response and result in a chronic inflammatory phenotype. A more comprehensive understanding of these sites and how they interact with their environment could eventually provide a means for therapeutic intervention.

7.2.1 Methylation of TNF

The findings of this research fit within the existing literature as regards the methylation of *TNF*, the associations with circulating fatty acids and *TNF* gene expression. Previously reported research, and the research that has been presented here indicates that some of these interactions are site specific, whilst others involve the methylation of multiple sites. Previous research had specifically found that two out of 20 CpG sites studied had a significant difference between high and low truncal fat and that the same sites were associated with gene expression. The assay used in this thesis measured four of the CpG sites from the aforementioned research. When analysing the relationship between circulating fatty acids and the methylation of these four sites (Chapter 4) the associations were not site specific; however consistent with previously reported results Hermsdorff *et al.* (2013), the methylation of CpG2 was the only site studied in this research to show association with anthropometric

measurements of body fat and BMI (Chapter 6). This site specifically, as noted by Hermsdorff *et al.* (2013), lies within the sequence RCGTG, which is the core binding motif for Hypoxia inducible factor (HIF-1), as shown below:

TNF exon 1: 5'-C**CG**GGA**CG**TGGAGCTGGC**CG**AGGAGG**CG**CT-3'

Therefore, altered methylation at this site could influence the binding of the HIF-1 transcription factor, thus influencing the expression of the gene. Methylation of this motif within the erythropoietin gene has been shown to prevent the binding of HIF-1 (Wenger *et al.*, 1998). However, it must be noted that, chromatin immunoprecipitation couple to next-generation high throughput sequencing (CHIP-seq) data investigating the binding sites of HIF-1 throughout the genome did not identify *TNF* as a target gene (Schodel *et al.*, 2011). Despite this, this individual CpG site (CpG2) has been found to be associated with measurements of obesity in both this research and that of Hermsdorff *et al.* (2013). Furthermore, methylation of the promotor and the first exon of *TNF* have been implicated in diseases such as cancer, where methylation of these regions is significantly associated with the suppression of *TNF* in tumours (Zhang *et al.*, 2009; Ganapathi *et al.*, 2014). Whilst there was no influence of ω-3 PUFA supplementation on the methylation of the four sites, the demethylation as a result of the exercise is an interesting discovery and investigating the impact of multiple bouts of exercise, such as an exercise training intervention, on the resting methylation levels of these sites warrants further investigation.

7.2.2 Methylation of IL1B

Our research has added to the limited research of the *IL1B* promotor. Although methylation of the *IL1B* gene was not altered as a result of ω-3 PUFA supplementation, EE induced an increase in the methylation of the sites studied. The methylation within the promotor of the *IL1B* gene is sparse, with no CpG island. Methylation of the *IL1B* promotor has been found to have a direct suppressive effect on expression of the gene in chondrocytes (Hashimoto *et al.*, 2013; Tekpli *et al.*, 2013; Kirchner *et al.*, 2014). The available research has predominantly focused on the methylation levels of the CpG sites located proximal to the TSS (-299bp) compared the region studied within this thesis, circa. -1000bp from the TSS. As explained in Chapter 3 this research was unable to validate an assay that would reliably measure the methylation of CpG site -

299bp from the TSS. The methylation of *IL1B* in chondrocytes is of particular relevance in rheumatoid arthritis, which is characterised by overexpression of *IL1B*. As is the case with *TNF*, it would be interesting to see if repeated bouts of exercise, particularly those at a lower intensity than someone suffering from rheumatoid arthritis could manage, would alter the methylation of the *IL1B* to provide a therapeutic effect.

7.2.3 Methylation of IL6

Previous research into the epigenetic regulation of the *IL6* gene has indicated the importance of methylation in the regulation of its gene transcription (Poplutz *et al.*, 2014). The sites studied in this thesis are located in a region that remains closed and methylated during LPS stimulation. However, these sites have been associated with obesity and altered by zinc deficiency (Na *et al.*, 2015; Wong, Rinaldi and Ho, 2015). The methylation of other CpG sites within the promotor of *IL6* have been investigated in relation to diseases such as rheumatoid arthritis and asthma (Nile *et al.*, 2008; Baccarelli *et al.*, 2012).

7.2.4 Regulation of gene transcription

It is important to understand the scale of the interactions between genes, transcriptional regulation (pre-, during and post-), interactions between proteins and feedback loops. For example, the cytokines themselves have an effect on the levels of methylation within the genome, such as IL1β and TNFα decreasing the expression and activity of the TET enzyme and leading to a decrease in 5-hmC throughout the genome (Haseeb, Makki and Haqqi, 2014) and IL6 affecting the activity of DNMT1 (Hodge *et al.*, 2007). It was not within the scope of this research to measure any other epigenetic markers than DNA methylation. However the transcriptional regulation of the genes studied is not solely controlled through the methylation of these specific sites, or DNA methylation across the gene, but rather a combination of multiple factors including histone modifications (Sullivan *et al.*, 2007; Lee, Sahoo and Im, 2009; Yoza and McCall, 2011; Poplutz *et al.*, 2014).

Considering DNA methylation alone, there are multiple modifications in the chemistry of cytosine other than that of the heavily studied methylcytosine, these include hydroxymethylcytosine, formylcytosine and carboxycytosine. Analysis of these alternative modifications cannot currently be resolved through the bisulphite

sequencing methods. Methods to quantify these other cytosine modifications are being developed but currently not concordant with each other or available for whole genome use (Lauschke, Ivanov and Ingelman-Sundberg, 2017).

It would be interesting to investigate the effects of similar interventions to the ones used within this thesis on other epigenetic modifications such as these cytosine modifications, histone modifications and micro RNAs.

7.2.5 Responders and non-responders

Previously in this thesis, the issue of responders and non-responders was briefly mentioned in Chapter 1 in relation to how epigenetic pharmacogenomics uses the genome of an individual in order to establish if they are responders or non-responders to particular drugs and to establish what dosage is appropriate. The implication for research is that within any cohort there may be individuals that respond to an intervention and there may be others that do not.

In Chapter 2 conditionally essential fatty acids were mentioned. At the level of dietary intake that may be essential to one person, may not be to another and therefore the effect of this supplementation may for example be seen in one person but not in another. 5

7.3 Thesis Limitations

7.3.1 Target tissue

There are a number of issues that require careful consideration in the design and implementation of epigenetic analysis of candidate genes. Identification of the candidate gene and the biologically significant region within it presents the first hurdle. To elucidate pathways in detail requires careful study of the baseline position and measurements to be made at precise CpG sites before analysing many potential induced effects. When associations are observed these need to be carefully considered and verified before there can be confidence to say that a pathway has been established.

For practical, and sometimes ethical reasons, it is not always possible to directly sample the tissue which would be the most interesting to study. This is problematic because alternative tissues must be used and an attempt to relate the results to the tissue of interest. This is not easily done because, as pointed out earlier in the thesis, methylation is specific to tissue type and responses to environmental changes differ according to cell type, so measurement of changes in one cell type may not be representative of the changes taking place in another cell type. Circulating blood comprises a heterogeneous cell population and this presents a barrier to interpretation of results. Different cell types within the blood complicate the position as they may have different methylation levels and may respond differently to intervention. It is recognised that tissue composition must be accounted for and that changes to tissue composition as a result of an intervention may lead to critical results being masked or exaggerated without specificity to one cell type or adjustment for any change (Jenke *et al.*, 2013).

In part this problem was addressed by performing a cell count for each sample collected which enabled the use of statistical correction to the methylation values to address the potential confounding factor of changes to tissue composition which arose from the inflammatory stimulus and resulted in a non-uniform increase in the different white blood cells. However, the white blood cells themselves comprise different cell types and future investigations would benefit from collecting a homogenous cell population; for example, by utilising the fluorescence-activated cell sorting (FACS)

method to obtain individual sub-populations of blood mononuclear cells. The use of FACS however also has its limitations and its use is restricted in studies such those carried out in this thesis because it would require a larger volume of blood to separate out the cells, and so sampling at a number of time points would not be possible.

7.3.2 Gene expression data

Many of the nutri-epigenetics studies are deficient in that they lack gene expression and protein measurement data and so make it difficult to investigate the biological significance of changes to DNA methylation. In Chapter 6 the methylation data was bilt upon by measuring mRNA expression and protein levels alongside the levels of methylation.

Unfortunately, due to the quality of the mRNA extracted from whole blood, the gene expression data proved difficult to analyse. In light of this, a more suitable approach for the collection of samples for RNA extraction would be collection of the buffy coat and preservation in TRIzol, rather than the whole blood. By using whole blood very few cells that contained RNA were captured within the sample, whereas the buffy coat would have concentrated the cells containing the RNA into the same volume of TRIzol. During design and implementation of the exercise protocol, it was considered that additional sample handling would not have been possible due to the pressure of the sampling schedule. In hindsight, it would have been better to reduce the number of samples collected (not all timepoints were analysed in this thesis) and to subject the smaller number of samples to additional processing in order to collect the buffy coat.

7.3.3 Cohort size and selection – power to detect changes

A common theme amongst intervention studies is the small size of the cohort population. This issue partly arises as a result of recruitment issues but the high costs of running the experiments and analysing results is also a major factor. Some epigenetic responses are sex-specific and this adds to the problem of obtaining a sufficiently large cohort to allow for stratification by sex.

The cohort size in Chapter 6 was halved for the second part of the research due to the split of participants by supplement type. Ideally, a larger cohort would have been used

so that after splitting the effect of any missed data points or outliers would not have impacted on the study.

7.3.4 Supplementation length

The supplementation periods in the second two studies were relatively short and no significant effects were seen. Longer periods of supplementation have their own issues as regards cohort recruitment, compliance and cost, however before it can be concluded with certainty that supplementation has no effect then longer interventions, even those that span generations are required.

7.4 Future directions

This research has proved promising in identifying changes to DNA methylation of candidate inflammatory genes. Future research is required to interrogate the observed changes in DNA methylation as the result of exercise and whether multiple bouts of reduced intensity exercise could provide a long-term beneficial change to DNA methylation profile of the genes. Furthermore, the relationship between fatty acids and DNA methylation could be interrogated further by using a different method of stimulation. As part of the research within Chapter 5, PBMCs were extracted before, during and after the ω-3 PUFA supplementation for *ex vivo* stimulation of the cells. Unfortunately, due to time pressures the method development for the *ex vivo* stimulation was not complete for inclusion in this thesis. It will be interesting to see if the methylation profiles of the cytokine genes respond to *ex vivo* stimulation in the same manner as the *in vivo* stimulation induced by EE.

This research did not show that increasing the dietary intake of EPA and DHA had any significant effect on the methylation of specific CpG sites within three cytokine genes. However, it did demonstrate that high intensity eccentric exercise altered methylation levels within these genes. The research here cannot be taken to indicate that changing dietary ω-3 PUFA has no effect on methylation throughout the genome, since at other CpG sites in other genes it may well have had an effect.

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Chapter 8 Appendices

Appendix 1: Data values for Figure 4-2 Spearman correlation coefficients heat map between the DNA methylation of four Cytosine-Guanine dinucleotides (CpG) sites located in exon 1 of the Tumor Necrosis Factor (TNF) gene and dried blood spot fatty acid levels measured as a percentage of total (spearman correlation coefficients)

	All										Males										Females									
	Raw (n=88)					Adjusted# (n=85)					Raw (n=30)					Adjusted# (n=29)					Raw (n=58)					Adjusted# (n=56)				
	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg
Omega 3 Polyunsaturated Fatty Acids (ω-3 PUFA)																														
20:5n-3 (EPA, Eicosapentaenoic acid)	-0.01	0.04	-0.01	-0.02	0.00	-0.01	0.03	0.01	-0.02	0.00	0.20	0.23	-0.06	0.06	0.11	0.25	0.31	-0.03	0.21	0.21	-0.10	-0.04	0.00	-0.06	-0.05	-0.12	-0.05	0.03	-0.08	-0.06
22:6n-3 (DHA, Docosahexaenoic acid)	0.00	0.13	0.12	0.06	0.09	-0.07	0.06	0.07	0.01	0.02	**0.49**	**0.51**	**0.38**	**0.47**	**0.50**	0.36	**0.44**	0.25	**0.38**	**0.39**	-0.24	-0.10	-0.06	-0.16	-0.15	**-0.35**	-0.19	-0.09	-0.26	-0.23
22:5n-3 (DPA, Docosapentaenoic acid	0.12	0.14	0.15	0.10	0.14	0.09	0.10	0.12	0.05	0.12	0.23	0.27	0.18	0.27	0.25	0.25	0.27	0.16	0.36	0.30	0.04	0.05	0.08	-0.01	0.05	-0.02	-0.02	0.06	-0.08	-0.01
18:3n-3 (ALA, alpha-Linolenic acid)	-0.10	-0.02	-0.07	-0.10	-0.08	-0.09	0.05	-0.04	-0.09	-0.03	-0.20	-0.22	-0.29	-0.32	-0.29	-0.14	-0.04	-0.16	-0.18	-0.17	-0.07	0.11	0.06	0.01	0.04	-0.09	0.18	0.06	0.03	0.07
TOTAL ω-3 PUFA	-0.03	0.04	0.02	-0.01	0.01	-0.05	0.05	0.02	-0.03	0.01	0.05	0.04	-0.06	-0.06	-0.02	0.04	0.17	-0.02	0.03	0.03	-0.08	0.02	0.06	0.02	0.02	-0.14	0.03	0.05	-0.03	-0.01
Omega 6 Polyunsaturated Fatty Acids (ω-6 PUFA)																														
18:3n-6 (GLA, γ-Linolenic acid)	-0.05	-0.17	-0.19	-0.08	-0.12	0.01	-0.16	-0.19	-0.04	-0.09	0.16	0.10	-0.01	0.19	0.09	0.19	0.09	-0.02	0.27	0.18	-0.16	**-0.34**	**-0.29**	-0.25	-0.26	-0.05	**-0.28**	-0.23	-0.16	-0.21
18:2n-6 (Linoelaidic acid)	-0.08	-0.18	-0.04	-0.16	-0.12	-0.12	-0.18	-0.08	-0.18	-0.14	-0.14	-0.11	-0.02	-0.13	-0.06	-0.03	0.00	0.06	0.04	0.04	-0.07	-0.22	-0.07	-0.19	-0.15	-0.17	-0.23	-0.14	-0.26	-0.21
20:4n-6 (AA, Arachidonic acid)	0.16	**0.24**	**0.28**	0.18	**0.23**	0.10	0.20	**0.26**	0.13	0.19	**0.37**	**0.47**	0.33	**0.38**	**0.44**	**0.45**	**0.57**	0.35	**0.46**	**0.49**	0.04	0.10	0.23	0.07	0.11	-0.06	0.03	0.19	-0.01	0.04
20:3n-6 (DGLA, Dihomo-γ-linolenic aci	-0.11	-0.04	0.03	-0.04	-0.05	-0.20	-0.06	-0.01	-0.08	-0.09	-0.18	-0.12	-0.14	-0.02	-0.13	-0.19	-0.11	-0.10	-0.01	-0.13	-0.07	0.05	0.15	-0.04	0.01	-0.18	0.05	0.11	-0.09	-0.05
20:2n-6 (Eicosadienoic acid)	0.12	0.12	0.16	0.18	0.16	0.15	0.16	0.19	**0.23**	**0.22**	0.02	-0.03	0.02	0.17	0.09	-0.07	-0.11	-0.09	0.12	0.01	0.18	0.22	**0.27**	0.21	0.24	0.22	**0.31**	**0.34**	**0.27**	**0.30**
22:2n-6 (Docosadienoic acid)	-0.07	-0.11	-0.05	-0.02	-0.07	0.00	-0.03	0.01	0.10	0.03	-0.06	-0.11	0.02	0.08	-0.08	-0.06	-0.15	-0.04	0.06	-0.08	-0.10	-0.15	-0.11	-0.12	-0.13	0.02	-0.08	-0.04	0.03	-0.03
22:4n-6 (Adrenic Acid)	**0.23**	**0.24**	**0.31**	**0.25**	**0.28**	0.13	0.17	**0.25**	0.15	0.20	0.21	0.32	0.28	0.35	0.31	0.24	0.28	0.24	0.33	0.30	0.25	0.22	**0.34**	0.22	**0.28**	0.15	0.13	**0.28**	0.14	0.20
TOTAL ω-6 PUFA	0.03	0.02	0.12	0.02	0.05	0.00	0.01	0.09	0.00	0.04	0.15	0.24	0.20	0.21	0.25	0.26	0.35	0.24	0.36	0.34	-0.03	-0.09	0.06	-0.10	-0.05	-0.11	-0.11	0.03	-0.15	-0.09
Monounsaturated Fatty Acids (MUFA)																														
14:1n-5 (Myristoleic acid)	-0.14	**-0.23**	-0.20	-0.13	-0.18	-0.14	-0.20	-0.21	-0.09	-0.18	**-0.45**	**-0.50**	**-0.37**	**-0.38**	**-0.48**	-0.35	**-0.47**	-0.30	-0.35	**-0.42**	0.04	-0.05	-0.06	0.04	0.00	-0.04	-0.09	-0.19	0.01	-0.09
16:1n-7 (Palmitoleic acid)	0.04	-0.05	-0.09	0.05	-0.02	0.11	0.04	-0.06	0.09	0.06	-0.26	**-0.36**	-0.31	-0.26	-0.33	-0.16	-0.28	-0.23	-0.16	-0.20	0.14	0.13	0.01	0.18	0.13	0.22	0.18	0.04	0.23	0.19
17:1n-7 (Cis-10-Heptadecenoic acid)	-0.05	-0.06	0.01	0.01	-0.02	-0.06	-0.05	0.04	0.01	-0.02	**-0.39**	-0.34	-0.21	-0.26	-0.35	-0.37	-0.34	-0.17	-0.31	-0.36	0.13	0.09	0.16	0.18	0.16	0.06	0.03	0.10	0.13	0.09
22:1n-17 (5Z-docosenoic acid)	0.10	0.07	0.12	0.17	0.12	0.01	0.02	0.07	0.11	0.05	-0.16	-0.24	-0.01	-0.04	-0.12	-0.18	-0.34	-0.08	-0.13	-0.20	0.21	0.23	0.20	**0.28**	0.23	0.12	0.15	0.11	0.18	0.14
18:1n-9 (Oleic acid)	0.04	0.06	0.03	0.06	0.05	0.07	0.13	0.08	0.09	0.09	-0.19	-0.23	-0.05	-0.22	-0.20	-0.14	-0.14	0.08	-0.25	-0.15	0.18	0.24	0.09	0.25	0.21	0.16	0.25	0.05	0.26	0.21
18:1n-9 (9-octadecenoic acid)	-0.07	-0.08	-0.12	-0.12	-0.09	-0.02	-0.03	-0.09	-0.05	-0.03	0.19	0.17	-0.01	0.07	0.12	0.18	0.23	0.02	0.14	0.16	-0.21	-0.24	-0.17	-0.26	-0.23	-0.11	-0.13	-0.08	-0.13	-0.13
20:1n-9 (Gondoic acid)	0.15	0.02	0.06	0.11	0.09	0.10	0.01	0.03	0.07	0.03	0.05	-0.07	0.04	0.04	0.02	0.04	-0.15	-0.01	-0.04	-0.03	0.22	0.11	0.09	0.18	0.15	0.19	0.07	0.04	0.13	0.11
TOTAL MUFA	-0.08	-0.14	-0.13	-0.07	-0.11	-0.06	-0.09	-0.12	-0.02	-0.08	**-0.46**	**-0.50**	-0.32	**-0.38**	**-0.47**	**-0.40**	**-0.46**	-0.23	**-0.40**	**-0.43**	0.14	0.09	0.03	0.15	0.12	0.10	0.07	-0.06	0.15	0.08
Saturated Fatty Acids (SFA)																														
13:0 (Tridecanoic acid)	0.11	0.17	0.11	0.09	0.12	0.17	0.21	0.18	0.11	0.19	0.07	0.05	0.06	-0.05	0.01	0.09	0.09	0.11	-0.10	0.03	0.10	0.22	0.09	0.16	0.15	0.18	**0.27**	0.20	0.19	0.25
14:0 (Myristic acid)	-0.14	-0.10	-0.15	-0.18	-0.16	-0.09	-0.05	-0.07	-0.15	-0.10	-0.21	-0.19	-0.19	-0.25	-0.26	-0.17	-0.05	-0.03	-0.21	-0.17	-0.12	-0.08	-0.15	-0.15	-0.12	-0.05	0.02	-0.07	-0.09	-0.06
15:0 (Pentadecanoic acid)	-0.14	-0.03	-0.02	-0.05	-0.06	-0.16	-0.03	0.00	-0.09	-0.07	-0.28	-0.14	-0.01	-0.21	-0.16	-0.31	-0.13	0.03	-0.26	-0.23	-0.05	0.03	-0.04	0.06	0.01	-0.05	0.06	0.00	0.05	0.02
16:0 (Palmitic acid)	0.01	0.00	-0.04	-0.01	-0.02	0.03	-0.05	-0.05	0.00	-0.03	0.34	0.25	0.16	0.27	0.27	0.21	0.03	-0.03	0.13	0.14	-0.14	-0.14	-0.14	-0.17	-0.17	-0.07	-0.15	-0.09	-0.12	-0.15
18:0 (Stearic acid)	0.13	0.14	0.09	0.14	0.13	0.13	0.09	0.05	0.13	0.10	**0.42**	0.34	0.27	0.36	**0.39**	0.32	0.13	0.06	0.29	0.28	0.01	0.05	0.02	0.02	0.01	0.07	0.05	0.06	0.07	0.06
20:0 (Arachidic acid)	0.10	0.15	0.10	0.08	0.12	0.06	0.08	0.05	0.01	0.06	0.15	0.21	0.11	0.04	0.16	0.14	0.17	0.10	0.12	0.16	0.07	0.10	0.04	0.08	0.08	0.07	0.04	0.05	0.06	0.10
22:0 (Behenic acid)	0.13	0.18	0.13	0.14	0.16	0.08	0.10	0.07	0.07	0.10	0.20	0.22	0.17	0.17	0.24	0.17	0.17	0.08	0.25	0.25	0.07	0.13	0.06	0.10	0.10	0.06	0.03	0.05	0.06	0.09
TOTAL SFA	-0.01	0.04	-0.06	-0.02	-0.02	0.04	0.01	-0.04	0.00	-0.01	0.36	0.28	0.20	0.25	0.29	0.22	0.06	0.04	0.10	0.15	-0.18	-0.06	-0.19	-0.16	-0.17	-0.04	-0.03	-0.07	-0.06	-0.06
Fatty Acid Ratios																														
MUFA : SFA	-0.08	-0.13	-0.08	-0.04	-0.08	-0.09	-0.08	-0.07	-0.02	-0.07	**-0.51**	**-0.51**	-0.32	**-0.39**	**-0.48**	**-0.38**	**-0.40**	-0.18	-0.33	**-0.38**	0.14	0.08	0.08	0.17	0.14	0.05	0.05	-0.04	0.12	0.06
PUFA : SFA	0.03	0.03	0.11	0.04	0.06	0.00	0.05	0.10	0.02	0.06	-0.02	0.09	0.06	0.04	0.06	0.12	0.29	0.18	0.24	0.20	0.05	0.00	0.14	0.04	0.07	-0.05	0.01	0.09	-0.03	0.01
MUFA&PUFA : SFA	0.01	-0.04	0.06	0.02	0.02	-0.04	-0.01	0.04	0.00	0.01	-0.36	-0.28	-0.20	-0.25	-0.29	-0.21	-0.06	-0.04	-0.10	-0.15	0.18	0.06	0.19	0.16	0.17	0.04	0.03	0.07	0.06	0.06
ω-6 PUFA : ω-3 PUFA	0.08	0.00	0.09	0.08	0.07	0.08	-0.02	0.07	0.08	0.05	0.10	0.18	0.28	0.29	0.25	0.12	0.07	0.19	0.25	0.20	0.08	-0.09	-0.02	-0.03	-0.03	0.13	-0.10	-0.01	-0.01	-0.02

Significant (p<0.05) Spearman's correlation coefficients between the four cytosine-guanine dinucleotide (CpG) sites (+197, +202, +214 and +222 base pairs from Transcription Start Site) of TNF gene and fatty acids detected in blood; average methylation of the four sites (CpGavg). Bold values indicate significant correlation (p<0.05). # CpG methylation percentage adjusted for white blood cell population.

Appendix 2: Example Participant Consent Form

Informed Consent Form

Loughborough University

INFORMED CONSENT FORM

Investigating the effect of 4-week n-3 PUFA supplementation on inflammation.

(to be completed after Participant Information Sheet has been read)

Taking Part	**Please initial box**
The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee.	☐
I have read and understood the information sheet and this consent form.	☐
I have had an opportunity to ask questions about my participation.	☐
I understand that I am under no obligation to take part in the study, have the right to withdraw from this study at any stage for any reason, and will not be required to explain my reasons for withdrawing.	☐
I agree to take part in this study.	☐
Use of Information	
I understand that all the personal information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others or for audit by regulatory authorities.	☐
I agree for the data I provide to be securely archived at the end of the project.	☐
Bodily Samples	
I agree that the bodily samples taken during this study can be stored until October 2019 for future research in the same research theme as this project.	☐
OR	
I agree that the bodily samples taken during this study can **only be** used for this study and will be disposed within 2 years.	☐

_____		_____	_____
Name of participant	[printed]	Signature	Date
_____		_____	_____
Researcher	[printed]	Signature	Date

Version 1

Appendix 3: PBMC cryopreservation

8.1.1 Sample Collection and Cell Separation

Cells used for this chapter were collected in Chapter 5, an additional four 10 mL tubes of blood were collected for this work. Whole blood samples were collected in EDTA coated vacutainers and stored at room temperature prior to processing, which occurred within a few hours of sample collection. Processing of blood was carried out in a class II microbiological flow hood under aseptic conditions PBMCs were extracted from the whole blood sample using Ficoll Plaque density gradient centrifugation, as described in section 5.2.5. After extraction, PBMCs were washed twice in hanks salt solution.

8.1.2 Cryopreservation

Extracted PBMCs were stored in media [Iscove's Modified Eagle Media (IMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/ml of penicillin and 10 µg/ml of streptomycin (PS)]. Each sample was stored with 10% Dimethyl sulfoxide (DMSO) in cryopreservation vials and frozen overnight in a "Mr Frosty" (Fisher Scientific, Loughborough, UK) at 1^{o}C/minute to -80^{o}C before being transferred to liquid nitrogen for cryopreservation until required.

8.1.3 Cell Culture and Treatments - Viability

Samples were rapidly thawed and suspended in Iscove's Modified Eagle Media (IMEM) (10% FBS and 1% PS) within a flask. To isolate the adherent cells within the sample, the flask was incubated at 37^{o}C at 5% CO_2 for 2 hours.

Non-adherent cells were removed with the media suspension. The adhered cells were released from the flask using Accutase (approx. 2mL) and washed with IMEM media (10% FBS and 1% PS) (2 x accutase volume). Cells were pelleted, and supernatant removed. The pelleted cells were re-suspended in 100uL IMEM media (10% FBS and 1% PS). Cell count completed using 1:1 Trypan Blue using a haemocytometer. Cells were seeded to a density of 100,000 cells per well in a 6 well plate lined with a collagen matrix. Collagen matrix was made up of 85% collagen, 10% MEM media and 5% cell

suspension; the collagen was neutralised with sodium hydroxide prior to the addition of the cell suspension. 300uL of collagen was set in the bottom of each well.

Cells maintained at 37^{o}C in 5% CO_2 with IMEM media (10% FBS and 1% PS) within a thermally regulated incubator.

8.1.3.1 *Cell viability*

The cell viability was measured by assessing the metabolic activity of the adhered cells and the dissociated cells using alamar Blue every 24 hours (approx.). A solution of alamar Blue (1:10) to IMEM media was used. Media removed from each well was kept and spun down to allow for analysis of the metabolic activity of the dissociated cells. The alamar Blue solution was added to each well and cell pellet. Cell incubated for 4 hours prior to fluorescence imaging.

8.1.3.2 *Imaging*

After collecting of cell viability data, cell density was capture using a light microscope. Cells per frame were quantified.

Appendix 4: Data values for Figure 6-15 (spearman correlation coefficients)

	Tumor necrosis factor (*TNF*) methylation (%)																			
	Pre-EE					*Post-EE*					*Post-EE+3h*					*Post-EE+48h*				
	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg	CpG1	CpG2	CpG3	CpG4	CpGavg
Interleukin 6 (*IL6*) methylation (%)																				
CpG1	-0.07	**-0.53**	**-0.66**	-0.49	**-0.51**	-0.44	-0.16	**-0.51**	**-0.55**	-0.44	-0.09	-0.25	-0.36	-0.38	-0.27	0.16	0.18	0.39	0.06	0.20
CpG2	0.14	0.01	-0.20	0.00	-0.04	0.01	-0.36	**-0.56**	**-0.63**	-0.47	0.06	-0.07	-0.12	-0.11	0.04	0.16	0.10	0.24	0.17	0.16
CpG3	-0.09	0.02	-0.13	-0.08	-0.08	-0.29	**-0.58**	-0.39	-0.24	-0.40	0.18	-0.01	0.17	0.00	0.13	-0.22	-0.19	-0.02	0.03	-0.08
CpG4	0.16	0.12	0.09	-0.09	0.02	0.47	0.34	-0.09	-0.10	0.06	0.17	-0.05	-0.05	0.05	0.03	**0.80**	**0.54**	0.48	**0.58**	**0.63**
CpG5	0.39	0.12	-0.15	0.14	0.10	-0.49	**-0.67**	-0.39	-0.48	**-0.51**	0.26	0.20	0.00	0.02	0.17	-0.24	-0.38	-0.14	-0.01	-0.13
CpG6	0.49	0.33	0.15	**0.51**	0.37	-0.37	-0.49	-0.42	**-0.50**	-0.44	0.42	0.20	0.15	0.10	0.24	-0.19	-0.28	-0.16	0.04	-0.11
CpGavg	0.38	0.13	-0.15	0.13	0.09	-0.35	-0.48	**-0.61**	**-0.69**	**-0.58**	0.31	0.11	-0.10	-0.01	0.12	-0.02	-0.10	0.10	0.15	0.06
Interleukin beta (IL1B) methylation (%)																				
CpG1	**-0.55**	**-0.53**	**-0.66**	**-0.62**	**-0.66**	0.21	-0.13	-0.37	-0.44	-0.20	0.26	0.07	0.17	0.03	0.15	0.04	-0.18	-0.04	-0.16	-0.10
CpG2	-0.34	-0.38	-0.38	-0.37	-0.40	0.05	-0.23	-0.34	**-0.52**	-0.28	-0.07	-0.31	-0.15	-0.21	-0.33	0.19	0.26	-0.15	0.14	0.03
CpG3	**-0.65**	**-0.57**	**-0.71**	**-0.81**	**-0.76**	0.22	0.06	-0.28	**-0.50**	-0.16	0.12	-0.19	-0.21	-0.13	-0.20	0.46	0.37	0.13	0.28	0.28
CpG4	**-0.65**	-0.47	**-0.55**	**-0.80**	**-0.69**	0.17	0.02	-0.29	-0.43	-0.17	0.02	-0.25	-0.20	-0.15	-0.28	-0.04	0.01	-0.43	-0.30	-0.27
CpG5	**-0.59**	**-0.66**	**-0.59**	**-0.75**	**-0.70**	0.11	-0.45	0.05	-0.17	-0.08	0.24	0.25	0.24	0.11	0.22	0.18	0.16	0.32	0.28	0.26
CpGavg	**-0.70**	**-0.63**	**-0.73**	**-0.83**	**-0.80**	0.28	0.01	-0.18	-0.40	-0.07	0.20	-0.02	0.00	0.02	0.02	0.15	0.11	-0.13	0.00	-0.03
Gene Expression																				
COX2	-0.45	-0.39	-0.30	-0.33	-0.38	-0.20	0.07	-0.27	-0.29	-0.20	-0.20	-0.18	-0.29	-0.32	-0.14	-0.24	-0.29	-0.19	-0.38	-0.34
DNMT3a	-0.13	-0.07	-0.15	-0.15	-0.12	-0.02	-0.05	-0.28	-0.03	-0.22	0.13	0.24	0.12	0.04	0.18	-0.27	-0.16	-0.07	-0.08	-0.17
DNMT3b	-0.08	0.00	0.05	-0.08	-0.02	0.13	0.26	-0.17	0.21	0.00	-0.04	0.06	-0.04	-0.05	0.05	-0.02	-0.15	0.07	0.00	0.01
IL1B	**-0.55**	-0.40	-0.19	-0.39	-0.36	-0.22	-0.22	-0.39	-0.30	-0.38	0.06	0.19	-0.01	0.07	0.03	**-0.50**	**-0.54**	-0.32	-0.42	-0.48
IL6	-0.25	-0.03	0.08	0.04	-0.02	0.13	0.24	-0.35	-0.29	-0.14	0.02	-0.07	-0.12	-0.07	-0.04	0.01	-0.06	0.24	0.23	0.14
PPARGC1A	0.09	0.09	0.10	0.00	0.07	-0.28	-0.24	**-0.64**	-0.30	**-0.51**	0.38	0.41	0.19	0.39	0.37	-0.26	-0.09	-0.09	-0.09	-0.16
TNF	-0.37	**-0.65**	**-0.63**	**-0.56**	**-0.64**	0.01	-0.03	-0.02	0.02	-0.07	0.22	0.24	0.28	0.07	0.17	-0.15	0.01	-0.04	0.06	-0.10
Serum Proteins																				
TNFa	-0.39	-0.36	-0.18	-0.26	-0.27	-0.28	-0.05	0.06	-0.07	-0.10	-0.18	-0.14	-0.36	-0.26	-0.10	**0.59**	**0.62**	0.37	0.32	0.43
IL6	0.26	-0.09	-0.22	0.19	0.04	-0.09	0.23	-0.21	-0.16	-0.06	-0.35	-0.15	-0.28	-0.13	-0.20	0.01	0.02	-0.17	-0.15	-0.14
LDH	0.00	0.13	-0.05	0.09	0.07	0.21	0.18	-0.12	-0.19	-0.02	**-0.52**	-0.49	-0.23	-0.30	-0.28	-0.10	-0.20	0.11	-0.05	-0.04
CK	0.31	**0.84**	**0.63**	0.46	**0.60**	0.00	0.04	-0.18	-0.08	-0.16	-0.41	-0.09	-0.15	-0.03	-0.07	-0.12	-0.19	0.04	-0.10	-0.18
Myoglobin	-0.43	-0.08	-0.03	-0.19	-0.16	0.05	0.14	0.01	-0.14	0.02	-0.37	-0.16	-0.14	-0.11	-0.11	-0.04	-0.21	0.18	-0.02	-0.05
Anthropometrics																				
Weight (Kg)	-0.25	-0.35	-0.09	-0.26	-0.23	-0.15	-0.32	-0.32	-0.17	-0.27	0.47	**0.54**	0.37	**0.54**	0.42	-0.38	-0.34	-0.29	-0.30	-0.35
Body Fat (Kg)	-0.48	**-0.55**	-0.28	-0.46	-0.46	-0.17	-0.12	-0.24	-0.11	-0.17	0.42	**0.51**	0.30	**0.52**	0.33	-0.43	-0.35	-0.27	-0.42	-0.39
Body Fat (%)	**-0.59**	**-0.58**	-0.32	**-0.53**	**-0.54**	-0.11	0.03	-0.17	-0.02	-0.07	0.37	0.47	0.23	0.45	0.27	-0.40	-0.33	-0.21	-0.40	-0.34
Water weight (%)	0.49	**0.59**	0.30	0.47	0.49	0.17	-0.01	0.25	0.14	0.15	-0.42	-0.46	-0.19	-0.41	-0.24	0.34	0.28	0.26	0.37	0.33
BMI (m/kg^2)	-0.38	**-0.51**	-0.22	-0.38	-0.38	-0.09	-0.15	-0.20	-0.11	-0.13	0.47	**0.52**	0.34	**0.54**	0.37	-0.41	-0.32	-0.32	-0.41	-0.38

Significant ($p<0.05$) Spearman's correlation coefficients shown in bold. Methylation of cytosine-guanine dinucleotide (CpG) sites for TNF; average methylation of the four sites (CpGavg) Before (Pre), and after (Post-EE, Post-EE+3h and Post-EE+48h) Eccentric Exercise (EE).

	Interleukin 6 (*IL6*) methylation (%)																												
	Pre-EE							*Post-EE*								*Post-EE+3h*								*Post-EE+48h*						
	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpGavg	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpGavg	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpGavg	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpGavg
Interleukin beta (IL1B) methylation (%)																													
CpG1	0.22	**0.69**	**0.71**	0.23	0.05	0.09	0.45	**0.50**	**0.88**	0.39	0.34	0.30	**0.62**	**0.72**	**0.60**	0.42	**0.50**	-0.01	0.34	0.39	**0.52**	**0.61**	**0.72**	**0.54**	0.12	0.43	0.34	**0.70**
CpG2	0.00	0.38	0.47	-0.11	0.01	-0.04	0.20	**0.57**	**0.77**	0.32	0.22	**0.52**	**0.75**	**0.78**	0.37	-0.02	0.20	**0.50**	-0.22	0.00	0.07	0.22	**0.54**	0.19	-0.01	0.22	0.35	0.41
CpG3	0.35	0.36	0.39	0.16	-0.05	-0.17	0.19	0.45	**0.75**	0.02	**0.50**	0.18	0.46	**0.58**	0.50	0.48	0.34	0.21	-0.07	0.04	0.24	0.46	**0.66**	0.27	0.17	0.32	0.33	**0.52**
CpG4	0.33	0.11	0.16	0.32	-0.21	-0.28	-0.02	**0.51**	**0.69**	0.10	0.40	0.19	**0.54**	**0.58**	0.48	0.24	0.38	0.28	-0.07	0.05	0.21	0.26	0.21	-0.08	-0.11	0.05	0.12	0.08
CpG5	0.28	-0.03	0.02	-0.09	-0.18	-0.34	-0.10	-0.02	0.36	0.05	-0.13	0.17	0.16	0.13	0.12	0.21	0.20	-0.35	0.31	0.03	0.15	0.39	**0.75**	**0.63**	-0.20	0.44	0.33	**0.66**
CpGavg	0.32	0.29	0.34	0.10	-0.12	-0.19	0.12	0.47	**0.74**	0.09	0.40	0.21	**0.53**	**0.58**	**0.62**	0.49	0.40	0.13	0.14	0.18	0.41	**0.51**	**0.72**	0.43	-0.05	0.38	0.37	**0.61**
Gene Expression																													
COX2	-0.12	-0.16	0.06	-0.12	-0.38	-0.31	-0.41	0.16	0.22	-0.27	0.10	0.10	-0.14	0.10	-0.15	0.07	0.02	-0.03	0.13	-0.08	0.04	-0.01	0.15	0.07	-0.06	-0.09	-0.24	0.02
DNMT3a	0.05	0.13	0.18	0.01	0.18	0.02	0.09	-0.31	0.24	0.00	0.46	-0.24	-0.32	-0.11	-0.36	-0.27	0.09	-0.13	0.12	0.02	0.00	-0.49	-0.42	-0.14	-0.06	-0.23	-0.31	-0.31
DNMT3b	-0.08	-0.02	-0.01	-0.09	-0.02	-0.25	-0.13	-0.36	-0.04	-0.05	0.39	-0.42	-0.46	-0.29	-0.28	-0.31	0.24	-0.06	0.14	0.03	0.01	-0.45	-0.33	-0.30	0.15	-0.11	-0.23	-0.33
IL1B	-0.11	-0.31	-0.13	-0.19	-0.43	-0.43	**-0.57**	-0.02	0.26	-0.05	0.03	0.08	-0.01	0.17	-0.31	0.05	-0.25	-0.23	-0.16	-0.45	-0.21	-0.35	-0.24	0.10	-0.08	0.06	-0.06	-0.06
IL6	-0.49	0.13	0.25	-0.14	-0.29	-0.05	-0.17	0.42	0.48	-0.17	0.42	-0.03	0.20	0.35	-0.44	-0.30	-0.04	0.18	-0.05	0.19	-0.10	-0.49	-0.28	-0.19	0.24	-0.03	-0.17	-0.17
PPARGC1A	0.13	0.23	0.15	0.46	0.01	0.09	0.15	0.00	0.34	0.44	0.22	-0.04	0.10	0.28	-0.19	-0.12	0.00	0.27	0.10	0.13	0.22	-0.42	-0.30	-0.17	-0.17	-0.27	-0.37	-0.30
TNF	0.47	0.04	-0.17	0.00	0.20	-0.15	0.12	**-0.65**	-0.02	-0.14	0.32	-0.33	-0.42	-0.32	-0.19	0.00	0.22	-0.10	-0.01	0.03	-0.01	-0.49	-0.44	-0.15	-0.01	-0.24	-0.19	-0.26
Serum Proteins																													
TNFa	-0.20	-0.19	0.04	-0.39	-0.48	-0.10	-0.40	-0.11	-0.34	-0.35	-0.07	0.03	-0.11	-0.13	0.01	0.02	-0.19	-0.09	0.22	0.04	0.09	0.11	-0.12	**-0.50**	0.50	**-0.67**	**-0.68**	-0.50
IL6	0.28	**0.54**	0.32	-0.08	0.22	**0.57**	**0.50**	0.23	0.11	0.11	-0.19	-0.09	0.00	0.10	0.09	0.07	-0.48	-0.05	-0.08	-0.37	-0.06	-0.16	0.05	-0.01	0.09	-0.34	-0.37	-0.15
LDH	-0.19	0.36	0.35	-0.08	0.10	0.11	0.28	0.10	0.32	-0.03	0.43	0.02	0.07	0.22	0.27	0.22	0.18	-0.10	0.10	-0.02	0.12	0.25	-0.07	-0.02	0.04	0.10	0.13	0.04
CK	-0.45	-0.03	0.09	0.29	0.01	0.17	0.09	-0.11	0.14	-0.06	0.43	-0.10	-0.32	-0.11	-0.28	-0.09	**-0.53**	-0.37	-0.03	-0.45	-0.27	-0.15	0.00	0.02	0.12	-0.04	-0.11	0.05
Myoglobin	-0.16	-0.46	-0.32	**-0.50**	-0.21	**-0.63**	**-0.55**	0.00	0.13	-0.17	-0.03	-0.10	-0.25	-0.08	-0.19	0.16	-0.43	-0.27	-0.11	-0.39	-0.20	0.12	0.25	0.02	0.19	0.26	0.23	0.30
Anthropometrics																													
Weight (Kg)	0.01	-0.01	0.11	0.25	-0.23	-0.45	-0.18	0.20	0.35	0.49	0.02	0.25	0.27	0.43	-0.28	0.13	-0.17	-0.25	0.09	-0.11	-0.04	-0.04	0.16	0.17	-0.22	0.11	0.12	0.16
Body Fat (Kg)	0.08	-0.10	-0.01	0.18	-0.27	-0.41	-0.26	0.22	0.11	0.36	-0.11	0.12	0.17	0.32	-0.23	0.04	-0.25	-0.14	-0.03	-0.22	-0.10	0.09	0.10	0.00	-0.32	-0.07	-0.09	0.00
Body Fat (%)	0.03	-0.08	0.08	0.20	-0.31	-0.41	-0.29	0.13	0.02	0.21	-0.09	-0.04	0.00	0.17	-0.32	0.02	-0.28	-0.12	-0.14	-0.33	-0.20	0.01	0.01	-0.08	-0.25	-0.19	-0.24	-0.11
Water weight (%)	-0.04	0.00	-0.13	-0.22	0.26	0.34	0.20	-0.22	-0.11	-0.26	0.05	-0.03	-0.13	-0.28	0.20	-0.11	0.28	0.05	0.10	0.26	0.12	0.00	-0.06	0.09	0.21	0.15	0.16	0.06
BMI (m/kg^2)	0.06	-0.03	0.01	0.14	-0.24	-0.41	-0.21	0.16	0.18	0.46	-0.07	0.16	0.23	0.36	-0.19	0.20	-0.13	-0.19	-0.01	-0.19	-0.07	0.04	0.05	-0.01	-0.32	-0.09	-0.07	-0.04

Significant ($p<0.05$) Spearman's correlation coefficients shown in bold. Methylation of cytosine-guanine dinucleotide (CpG) sites for *IL6*; average methylation of the four sites (CpGavg) Before (Pre), and after (Post-EE, Post-EE+3h and Post-EE+48h) Eccentric Exercise (EE).

	Interleukin beta (*IL1B*) methylation (%)																							
	Pre-EE						*Post-EE*						*Post-EE+3h*						*Post-EE+48h*					
	CpG1	CpG2	CpG3	CpG4	CpG5	CpGavg	CpG1	CpG2	CpG3	CpG4	CpG5	CpGavg	CpG1	CpG2	CpG3	CpG4	CpG5	CpGavg	CpG1	CpG2	CpG3	CpG4	CpG5	CpGavg
Gene Expression																								
COX2	0.18	0.03	0.21	0.22	0.04	0.25	-0.06	-0.04	0.01	-0.13	-0.19	-0.08	-0.02	-0.14	-0.10	-0.05	0.28	-0.04	0.26	-0.11	-0.15	0.05	0.00	0.00
DNMT3a	0.09	-0.19	0.14	0.06	-0.20	0.07	-0.04	-0.16	-0.09	-0.15	-0.20	-0.19	-0.22	-0.26	-0.33	-0.26	-0.03	-0.34	-0.40	-0.33	**-0.56**	-0.37	-0.30	-0.49
DNMT3b	-0.03	-0.21	-0.01	-0.02	-0.20	-0.06	-0.15	-0.30	-0.20	-0.27	-0.43	-0.34	-0.34	-0.23	-0.34	-0.23	-0.23	-0.40	-0.35	-0.28	-0.23	-0.34	-0.07	-0.34
IL1B	0.10	0.06	0.22	0.34	0.18	0.27	0.06	0.13	0.11	-0.01	0.15	-0.04	-0.02	-0.01	0.09	0.10	0.35	0.10	0.02	-0.41	**-0.60**	-0.23	-0.38	-0.40
IL6	0.15	-0.12	-0.07	-0.15	-0.28	-0.07	**0.55**	0.49	**0.56**	**0.51**	-0.10	0.47	**-0.58**	-0.39	-0.48	-0.45	-0.41	**-0.63**	-0.35	-0.22	-0.32	-0.46	-0.04	-0.39
PPARGC1A	-0.02	**-0.52**	-0.05	0.05	-0.43	-0.15	0.20	0.02	-0.06	-0.04	-0.32	-0.14	-0.11	-0.09	-0.14	0.00	-0.14	-0.01	-0.44	-0.15	-0.37	-0.15	-0.03	-0.31
TNF	0.35	0.12	0.39	0.31	0.39	0.41	-0.27	-0.19	-0.14	-0.25	-0.09	-0.26	0.06	-0.10	-0.06	-0.05	0.18	0.02	-0.39	-0.28	**-0.55**	-0.40	-0.40	**-0.50**
Serum Proteins																								
TNFa	0.12	0.37	0.33	0.24	**0.50**	0.36	-0.48	-0.10	-0.06	-0.05	-0.21	-0.13	-0.27	-0.49	-0.34	**-0.53**	-0.05	-0.38	-0.26	0.03	0.14	0.11	-0.02	-0.06
IL6	0.28	0.08	-0.03	-0.19	-0.13	-0.04	0.19	-0.11	-0.13	-0.19	0.00	-0.15	-0.27	-0.19	-0.20	-0.21	-0.13	-0.13	0.11	-0.03	-0.22	-0.19	0.06	-0.13
LDH	0.25	0.19	0.21	0.05	0.05	0.19	0.29	0.44	**0.59**	**0.54**	-0.13	**0.51**	-0.03	-0.05	-0.08	-0.10	-0.14	-0.06	0.01	0.10	0.11	0.15	-0.20	0.16
CK	-0.31	-0.31	-0.26	-0.11	-0.36	-0.31	-0.11	-0.14	-0.05	-0.12	-0.25	-0.13	-0.40	-0.48	**-0.51**	-0.50	0.05	-0.41	0.16	-0.24	-0.27	-0.41	-0.03	-0.25
Myoglobin	-0.26	-0.26	-0.15	-0.15	0.00	-0.15	0.05	-0.11	-0.13	-0.29	0.21	-0.14	-0.16	-0.30	-0.28	-0.24	0.08	-0.14	0.38	-0.05	-0.06	-0.19	0.01	-0.08
Anthropometrics																								
Weight (Kg)	0.11	-0.16	-0.12	-0.09	0.00	-0.11	0.29	0.14	-0.03	-0.06	0.13	0.02	-0.05	-0.24	-0.03	-0.10	0.41	0.03	0.28	0.05	-0.27	-0.26	0.01	-0.13
Body Fat (Kg)	0.22	-0.06	0.07	0.13	0.21	0.11	0.14	-0.03	-0.16	-0.18	-0.01	-0.13	-0.05	-0.06	0.06	0.08	0.33	0.13	0.23	-0.02	-0.28	-0.13	-0.03	-0.14
Body Fat (%)	0.30	0.03	0.17	0.23	0.24	0.22	0.04	-0.18	-0.25	-0.28	-0.08	-0.23	-0.10	-0.02	0.07	0.13	0.30	0.11	0.14	-0.12	-0.38	-0.18	-0.07	-0.24
Water weight (%)	-0.32	-0.10	-0.16	-0.18	-0.26	-0.20	-0.15	0.05	0.12	0.14	0.05	0.11	0.04	-0.04	-0.17	-0.18	-0.31	-0.18	-0.20	-0.02	0.28	0.08	0.10	0.14
BMI (m/kg^2)	0.22	-0.01	0.03	0.06	0.17	0.07	0.22	0.05	-0.10	-0.14	0.05	-0.06	-0.01	-0.10	0.12	0.09	0.32	0.17	0.17	0.03	-0.28	-0.11	-0.08	-0.14

Significant ($p<0.05$) Spearman's correlation coefficients shown in bold. Methylation of cytosine-guanine dinucleotide (CpG) sites for *IL1B*; average methylation of the four sites (CpGavg) Before (Pre), and after (Post-EE, Post-EE+3h and Post-EE+48h) Eccentric Exercise (EE).

Appendix 5: Fatty acid profile in whole blood (Chapter 4) vs. peripheral blood mononuclear cells (Chapter 5)

The fatty acid profile of whole blood which was measured from dried blood spots in Chapter 4 was compared to the fatty acid profile at baseline of the white blood cells. The percentage total fatty acids differed for all bar a few fatty acids.

Table 8-1 Comparison of fatty acid profile in whole blood (from dried blood spots) and white blood cells.

	Whole Blood n = 30	White Blood Cells n = 17	*p*
Omega 3 Polyunsaturated Fatty Acids (ω-3 PUFA)			
18:3ω-3 (ALA, alpha-Linolenic acid)	5.31 ± 1.13	7.04 ± 0.84	**< 0.001**
20:5ω-3 (EPA, Eicosapentaenoic acid)	0.47 ± 0.22	0.09 ± 0.05	**< 0.001**
22:5ω-3 (DPA, Docosapentaenoic acid)	0.63 ± 0.14	0.43 ± 0.18	**< 0.001**
22:6ω-3 (DHA, Docosahexaenoic acid)	1.22 ± 0.47	0.51 ± 0.18	**< 0.001**
TOTAL ω-3 PUFA	7.63 ± 1.36	8.06 ± 1.08	0.268
Omega 6 Polyunsaturated Fatty Acids (ω-6 PUFA)			
18:2ω-6 (LA, Linoleic acid)	13.69 ± 1.62	2.64 ± 0.87	**< 0.001**
18:3ω-6 (GLA, γ-Linolenic acid)	1.09 ± 0.42	0.61 ± 0.29	**< 0.001**
20:2ω-6 (Eicosadienoic acid)	0.79 ± 0.24	0.45 ± 0.21	**< 0.001**
20:3ω-6 (DGLA, Dihomo-γ-linolenic acid)	6.54 ± 1.49	5.78 ± 2.07	0.155
20:4ω-6 (AA, Arachidonic acid)	0.43 ± 0.13	0.39 ± 0.15	0.343
22:4ω-6 (Adrenic Acid)	0.14 ± 0.06	0.15 ± 0.07	0.828
22:2ω-6 (Docosadienoic acid)	0.10 ± 0.03	0.44 ± 0.15	**< 0.001**
TOTAL ω-6 PUFA	22.79 ± 2.79	10.45 ± 3.00	**< 0.001**
Monounsaturated Fatty Acids (MUFA)			
14:1ω-5 (Myristoleic acid)	14.06 ± 2.42	13.36 ± 5.09	0.601
16:1ω-7 (Palmitoleic acid)	1.05 ± 0.23	3.43 ± 3.21	**0.008**
17:1ω-7 (Cis-10-Heptadecenoic acid)	2.33 ± 0.86	2.24 ± 0.91	0.742
22:1ω-17 (5Z-docosenoic acid)	0.60 ± 0.12	0.32 ± 0.15	**< 0.001**
18:1ω-9 (Oleic acid)	2.87 ± 0.99	8.29 ± 3.16	**< 0.001**
18:1ω-9 (9-octadecenoic acid)	0.71 ± 0.36	0.43 ± 0.22	**0.002**
TOTAL MUFA	22.00 ± 3.53	28.08 ± 9.97	**0.026**
Saturated Fatty Acids (SFA)			
13:0 (Tridecanoic acid)	0.08 ± 0.04	1.38 ± 0.32	**< 0.001**
14:0 (Myristic acid)	3.25 ± 1.22	3.83 ± 1.85	0.199
15:0 (Pentadecanoic acid)	0.95 ± 0.33	2.52 ± 1.18	**< 0.001**
16:0 (Palmitic acid)	23.01 ± 2.00	25.77 ± 4.85	**0.037**
18:0 (Stearic acid)	19.44 ± 1.36	19.20 ± 3.73	0.801
20:0 (Arachidic acid)	0.45 ± 0.28	0.44 ± 0.21	0.970
22:0 (Behenic acid)	0.41 ± 0.13	0.20 ± 0.06	**< 0.001**
TOTAL SFA	47.59 ± 2.70	53.41 ± 8.84	**0.017**
Fatty Acid Ratios			
MUFA / SFA	0.47 ± 0.09	0.57 ± 0.31	0.188
PUFA / SFA	0.64 ± 0.09	0.35 ± 0.08	**< 0.001**
(MUFA+PUFA) / SFA	1.11 ± 0.12	0.92 ± 0.34	**0.043**
ω-3 PUFA / ω-3 PUFA	3.06 ± 0.56	1.28 ± 0.28	**< 0.001**
AA / EPA	16.28 ± 7.73	14.95 ± 14.08	0.677

Data is presented as Percentage Total Fatty Acids (%TFA), mean ± standard deviation. Significant difference assessed by t-tests, significant difference if $p<0.05$.

Appendix 6: Published Editorial

Editorial

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Epigenetics and epigenomics: the future of nutritional interventions?

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"Nutri-epigenomics looks to delineate the interactions between the diet and the genome through epigenetic mechanisms."

Keywords: DNA methylation • epigenetics • personalized nutrition

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For many years, it was thought that sequencing the human genome would hold the answer to health and disease. However, after years of research and billions of dollars of funding, the Human Genome Project provided us with the script for the human genome, but not the great leap in understanding for genetic medicine that was anticipated. We still have many questions, particularly how the genome interacts with the environment to result in disease in some individuals, but not in others. For this we need to focus on the epigenome. In the 1940s, the epigenetic landscape began to take shape; famously described by Waddington [1], it explained how cells with the same genetic code could differentiate into the vast array of cells within an organism, connecting the genotype with the phenotype. The field of 'epigenetics', literally meaning 'above genetics', investigates chemical modifications to DNA that do not alter the gene sequence itself [2]. Epigenetic mechanisms, such as chromatin remodeling, histone modifications, DNA methylation and microRNAs, alter how the genome is expressed. These modifications can be enduring and passed through generations, but they can also be transient, allowing cells to adapt to the environment. In recent years, there has been an exponential increase in the research and knowledge within the field of epigenetics. It is only now that we are beginning to piece together interactions between the genome and epigenome with the environment to comprehend the implications for health and disease. A key environmental factor that we are exposed to from preconception to death is our diet. Nutri-epigenomics looks to delineate the interactions between the diet and the genome through epigenetic mechanisms.

The susceptibility of the epigenome to adapt to environmental factors alters across the lifespan of an organism, during which there are periods when the epigenome is more responsive to change [3]. Increased activity of the epigenome occurs in the prenatal and neonatal phases when cell differentiation and specialization is occurring. Environmental factors, such as nutrition, therefore have more influence during this period of development. In nature this mechanism is utilized by the honeybee, they feed genetically identical larvae royal jelly to alter DNA methylation patterns with the result of differentiation to either a queen or a worker bee [4]. In humans, the Developmental Origins of Health and Disease hypothesis explains how abnormalities within the developmental environment can affect the writing of the epigenome and lead to metabolic disease later in life [5]. It is understood that maternal stress, birth weight and fetal malnutrition (undernutrition and maternal obesity) contribute to an abnormal developmental environment and modifications to the epigenome, and as a result, can be enduring throughout the lifespan and passed on to the next generation. In mammals, malnutrition of methyl donors vitamin B_{12} and choline during gestation can lead to epigenetic dysregulation within the offspring, leading to obesity and influencing disease in later life [6]. Providing a favorable developmental environment through the use of nutritional supplements or a specific gestational diet could result in beneficial epigenetic changes. For example,

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supplementation with omega-3 fatty acids during gestation has been shown to protect against demethylation caused by the negative behavior of smoking [7]. Looking to the future, is developing an 'epigenetically healthy' gestational diet the answer to reducing the burden of chronic disease in later life and within our offspring? Future research must consider the hereditary nature of epigenetic signatures, with studies requiring observation of multiple generations. There must also be consideration for the long-term epigenetic impact of ***in vitro*** fertilization (IVF), where the early stages of development receive nutrients from outside of the mother.

Convincing individuals to maintain an 'epigenetically healthy' diet in order to benefit the health of their children and grandchildren is often not effective as there is no immediate beneficial effect seen by the individual. Therefore, research into the short-term effects of diet on the epigenome is required in adults. Aberrant alterations to epigenetic regulation is implicated as the cause of multiple chronic diseases including cancer, chronic obstructive pulmonary disease, cardiovascular disease, obesity, neurological disorders and Type-2 diabetes, to name a few. These changes in the epigenome provide therapeutic targets for intervention. Drugs have been developed to target epigenetic regulating enzymes, such as DNMT, HDAC and HAT inhibitors in order to reverse the changes to the epigenome. Discussed in a mini-review series in *Clinical Epigenetics* [8], there is a huge potential for epigenetic drugs; however, there is a long way to go before we truly understand their impact, particularly their wider alterations to the epigenome. Nutritional interventions or dietary supplementation may therefore provide a safer approach to prevent or reset these aberrant changes by influencing the natural epigenetic response within cells.

Personalized medicine and interventions are now common with the use of genotyping an individual in order to distinguish if they are a responder or a nonresponder to drugs and nutritional interventions. There has also been an increase in the use of genetic testing to identify targeted populations and interventions that will reduce the burden of disease in later life [9]. However, there is building evidence that epigenetic signatures could also be used to personalize interventions and predict response status. For example, it has been found that determining an 'epigenotype' for an individual can predict whether they are going to be a weight-loss responder to a calorie restricted diet [10]. We therefore must also consider the effect of the epigenome on nutritional requirements. Data such as this may be used to combine both genotype and epigenotype of an individual to assess their risk status for disease and response to nutritional interventions.

Implementation of epigenetic analysis to nutritional studies has barriers that must be considered, including technical and cost limitations. Careful consideration must be taken when choosing the sample for epigenetic analysis. Epigenetic control of the genome is cell specific and conversely, this means that alterations in response to the environment will be individual to tissue type and to individual cells within the tissue. Therefore, selecting an easily accessible tissue such as blood for analysis may lead to misleading findings due to the heterogeneity of cells and care must be taken when inferring conclusions from the sample tissue to the desired tissue of interest. Additionally the tissue composition must be accounted for, changes to tissue composition as a result of an intervention may lead to critical results being masked or exaggerated without specificity to one cell type or adjustment for any change [11]. Establishing causation is often an issue in epigenetic research, although there have been statistical methods employed to delineate causation [12], and carefully designed and controlled research is vital. Considerations into the biological relevance of statistically significant results must be supported by phenotypic data such as gene expression and protein profiles. Epigenetic control of the genome is far more complicated than first thought with a multitude of different epigenetic mechanisms to quantify, plus their interactions with each other. For example, considering DNA methylation alone, there are multiple modifications in the chemistry to cytosine other than the heavily studied methylcytosine, including hydroxymethylcytosine, formylcytosine and carboxycytosine, that cannot currently be resolved through the bisulfite sequencing methods. Methods to quantify these other cytosine modifications are being developed but currently not concordant with each other or available for whole genome use [13]. Due to the complexity of the epigenome, many of the technologies used to measure its epigenetic makeup come at a high cost. Institutions and funding bodies must 'buy in' to running these high cost assays in order to gain additional information from their nutritional research.

Conclusion

Given the interactions between nutrition and the epigenome, future work in this field has the potential to provide a rewarding benefit for public health. If we want to understand how our body responds to nutritional interventions then we need to understand the interactions with the genome and epigenome. Understanding these modifications, which can be passed through generations, will provide vital knowledge to thwarting noncommunicable diseases and promoting healthy free living in later life. We have the potential to impact on the evolution of the human species by tailoring our diets to alter/maintain the epigenome.

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Appendix 7: Published Literature Review

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Omega 3 fatty acids, inflammation and DNA methylation: an overview

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ABSTRACT
Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are known to be anti-inflammatory and to alter gene expression within the cells. Emerging evidence indicates that one of the mechanisms for this process involves the alteration of epigenetic markers, such as DNA methylation. The focus of this overview is to document the current evidence for n-3 PUFA effects on DNA methylation and how these may impact on the inflammatory processes.

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Introduction

n-3 polyunsaturated fatty acids and inflammation

Populations with a diet rich in omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have a lower incidence of chronic non-communicable diseases [1]. Evidence for n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on their ability to modulate inflammation has come from *in vitro* work [2–4], animal models [5–8] and human studies [4,9,10]; although the latter evidence is weaker as a consequence of more complex environmental factors. The mechanisms through which n-3 PUFAs promote an anti-inflammatory environment within the body are multiple and complex [11] (Figure 1). Early evidence suggests that n-3 PUFAs exert these effects through changes in gene expression as a result of varied transcription factor activation [12,13], but which may also arise from changes in epigenetic markers, such as DNA methylation.

Epigenetics

Epigenetics investigates heritable chemical modifications to the genome which are independent of the DNA sequence [14–16]. These chemical changes, including DNA methylation, histone modifications, chromatin remodelling and non-coding RNAs [17–23], are vital for normal cellular development, cellular processes and cell-specific gene expression profiles [16,24]. Interactions between the environment, particularly diet, and the genome through epigenetic mechanisms lead to changes in phenotype and are implicated in many diseases [25–28]. They also provide a target for therapeutic interventions [29].

DNA methylation, a methyl group covalently attached to the fifth position of the pyrimidine ring to give 5-methylcytosine (5mC), is most commonly found on a cytosine positioned next to a guanine (CpG) [30]. DNA methylation levels within the genome are cell and tissue specific, with changes observed during the human lifespan [31–33]. Dysregulation of 5mC has been associated with diseases, including cancer and cardiovascular disease [24,34,35]. DNA methylation is the most accessible and widely studied epigenetic mark [36] and has been shown to vary with nutrition, disease and age [37–39]. Here, we will discuss the emerging evidence for the interaction of n-3 PUFAs and DNA methylation, specifically discussing their impact on inflammation.

Cross-sectional epigenome-wide association studies

Epigenome-wide associations studies (EWAS), which despite their name, focus mainly on DNA methylation, have linked n-3 PUFA consumption with differential DNA methylation. However, there is a lack of EWAS that use biochemical analysis to assess dietary n-3 PUFA, with a preference towards the readily available, but less accurate, food frequency questionnaire (FFQ).

One prominent EWAS within a distinct population, the *Yup'ik*, from Alaska (n = 185) utilised biochemical analysis to investigate associations with DNA methylation [40]. The

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Yup'ik have a traditional diet rich in fish which is changing with westernisation [41], allowing for stratification of this population into high and low PUFA consumption. Using red blood cell (RBC) nitrogen stable isotope (δ^{15} N), previously associated with EPA and DHA RBC levels [42], to separate into these high and low PUFA groups, associations with CpG methylation of biologically relevant targets, including genes involved in *T*-cell homeostasis were found [40]. Increased DNA methylation with high PUFA consumption was observed in 78% of the significant associations [40]; with genomic instability occurring with hypomethylation [43], the increased methylation observed may, therefore, be beneficial to the stability of the genome. A separate cohort, Greek pre-adolescents ($n = 69$), also found associations between dietary fats and DNA methylation, including sites within pathways linked to inflammation; nuclear factor kappa B (*NFκB*), peroxisome proliferator-activated receptor (*PPARα*), leptin (*LEP*) and interleukin (*IL*)-*6* [44]. However, the dietary fat intake for the cohort was assessed using FFQs, which did not directly measure the levels of n-3 PUFAs that have made it to the target tissue from the food.

Consistent with previous supplementation studies [45,46] and dietary salmon consumption [47], the *Yup'ik* cohort found individuals with higher n-3 PUFA intake had significantly lower levels of plasma triglyceride (TG), increased high-density lipoproteins (HDL), and higher but not significant levels of low-density lipoproteins (LDL) and total cholesterol [40]. However, Dekkers *et al.* [48] found that there was an effect of blood lipids (TG, HDL and LDL) on differential DNA methylation in the genes coding for six key regulators of lipid metabolism. It therefore, needs to be questioned if the differential methylation observed is due to the n-3 PUFAs in the diet, differences in blood lipid profiles, or both, and one method of addressing this is through closely controlled intervention studies.

Gestation and infant n-3 PUFA supplementation intervention studies

Epigenome-wide association studies

The impact of nutrition during early life is of increasing interest due to the plasticity of epigenetic regulation during development and the links to diseases in later life [49,50]. Supplementation during gestation or early infancy with n-3 PUFAs has therefore been studied to a greater extent than it has been in adults.

EWAS in combination pre-and post-intervention can be used to investigate the impact of n-3 PUFA supplementation on differentially methylated regions (DMRs) throughout the genome. Two intervention studies took slightly different approaches, the first supplemented the mothers with 3.7 g/day EPA + DHA ($n = 36$) or placebo ($n = 34$, placebo details not provided) from 20 weeks of gestation to delivery [51]. The second study supplemented

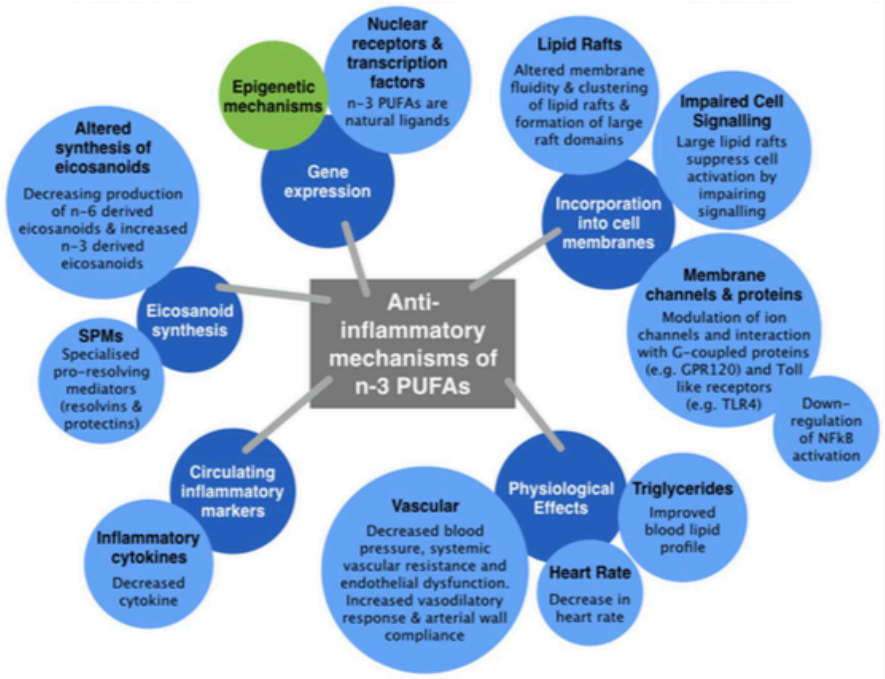

Figure 1. Mechanisms Omega-3 polyunsaturated fatty acids (n-3 PUFAs) action to promote an anti-inflammatory environment.
Notes: GPR120, G-coupled protein recptor 120; NFkB, nuclear factor kappa B; n-6 PUFAs, omega 6 polyunsaturated fatty acids; SPM, Specialised pro-resolving mediators; TLR4, toll like receptor 4.

9-month-old infants with either 1.6 g/day EPA + DHA ($n = 6$) or placebo 3.1 g/day linoleic acid ($n = 6$, LA in the form of sunflower oil) for nine months [52]. Neither investigation found a significant difference in DNA methylation between those taking the n-3 PUFA and those taking the placebo after adjusting for multiple testing [51,52].

However, a third more recent large randomised control trial (RCT) supplementing mothers during gestation with 0.8 g/day DHA + 0.1 g/day EPA ($n = 190$) or vegetable oil ($n = 179$), found that there were significant differences between DMRs, including those relating to immune function, between the two experimental groups [53]. The dose of n-3 PUFAs in this third study was much lower, in addition, due to the larger cohort it was possible to split the analysis between the sexes, with males being found to have a greater number of DMRs than females. The lack of findings in the first two studies referred to above may be due to the small, mixed sex samples used and future studies could be designed to eliminate this possibility. There are significant differences in the way males and females metabolise and store n-3 PUFAs [54–56] as well as differences in global methylation levels between the sexes [57]; as seen by the clear effect observed between sexes in the third study [53]. It is therefore, important to note that when using small sample sizes with n-3 PUFA and DNA methylation studies, a lack of separation of the sexes may result in critical results being overlooked.

Notably, all studies presented thus far have utilised the Infinium Human Methylation 450 k arrays. This technology measures DNA methylation at over 450,000 individual CpG sites throughout the genome. And although a powerful tool in explorative work in determining DMRs, there are limitations to the use of 450 k array data, including multiple testing, the need for complicated normalisation and the generation of artefactual data [58]. Therefore, targeted analysis of specific targets including candidate genes must be completed to confirm findings, and investigate differential methylation and biological relevance.

Specific targets and candidate genes

Long interspersed nucleotide element 1 (LINE1)

Measuring the levels of DNA methylation in repetitive elements, such as long interspersed nucleotide elements (LINEs) can provide a proxy for global DNA methylation [59]. It has been estimated that repetitive transposable elements account for 45% of the genome [60] and they contain one-third of the genomes DNA methylation [61,62] to repress their transcription [63]. Associations between *LINE1* methylation and lifestyle factors, including physical activity and nutrition [57,64–67] have been found and have also been shown to be associated with both disease [68–70] and ageing [71,72].

Lee *et al.* investigated the DNA methylation of *LINE1* in cord blood mononuclear cells (CBMCs) from mothers who received 0.4 g/day DHA ($n = 131$) or placebo ($n = 130$, olive oil) during gestation [73]. Neither this study or the third RCT above found any differences in *LINE1* methylation between supplementation and control groups [53,73]. However, Lee *et al.* did find an interaction between smoking status and DHA supplementation; CBMCs of mothers who smoked and took the DHA during gestation had significantly higher *LINE1* methylation levels than the CBMCs of smoking mothers in the control group [73]. This suggests a complex interaction between multiple environmental stimuli and the epigenome.

Cytokine genes

In addition to *LINE1* methylation, cytokine gene DNA methylation was also investigated by Lee *et al.*, with no significant differences in methylation of *TNFa*, *IL13*, *GATA3*, *STAT3*, *IL10* and *FOXP3* being observed. However, the promotor methylation was lower (not significantly) for *IFNy* in those supplemented with DHA [73]. The study was novel in that the supplementation only contained DHA and was also at a low dose compared to many other studies. As observed in cell membranes where there is a dose-response increase in the incorporation of n-3 PUFAs, it is likely that an increase in the dose may have led to more significant changes in DNA methylation. *In vitro* studies provide evidence of differing effects of EPA and DHA on inflammation [74,75], it would be interesting to see if there are differing effects on the epigenome had a separate EPA supplementation group had been included. The comparison between EPA and DHA is often lacking in human studies due to the increased cost and a greater availability of mixed n-3 PUFA supplements commercially.

Adult n-3 PUFA supplementation intervention studies

Candidate targets

PUFAs are important structural components of cells membranes. Consumption of foods rich in n-3 PUFAs, such as salmon, result in increased incorporation and modification of the cell phospholipid membrane [47]. Composition of the membrane is altered by clustering of lipids rafts containing the n-3 PUFAs, resulting in the formation of large raft domains [76]. As shown in murine obesity models [77] and HeLa cells [78] large lipid rafts can suppress the cell activation by impaired signalling, ultimately affecting cell function through suppression of downstream pathways, including those involved in inflammation. Leptin is localised to these lipid rafts and its expression is reduced by n-3 PUFAs [79]. In humans, cross-sectional evidence indicates

differential methylation in the leptin pathway genes with varying n-3 PUFA status [44], and epigenetic control of *LEP* has been demonstrated in a murine model [80]. However, using a candidate gene approach, n-3 PUFA supplementation has been shown to have no effect on *LEP*, leptin receptor (*LEPR*) or pro-opiomelanocortin (*POMC*) promotor methylation in a murine model [81].

The membrane glycoprotein which promotes inflammation in monocytes and macrophages, cluster of differentiation 36 (CD36), has been found to be increased as the result of n-3 PUFA *in vitro* [82] and in animals [83]. *CD36* promotor methylation was significantly reduced, when adjusted for baseline body weight, in a weight loss and n-3 PUFA supplementation study in Spanish young adult overweight females [84]. However, changes in DNA methylation in cluster of differentiation (*CD14*), pyruvate dehydrogenase kinase 4 (*PDK4*) and fatty acid desaturase 1 (*FADS1*) was only significant as a result of the low-calorie diet and not the supplementation. Had the gene expression been studied we may have expected to see an increase in the mRNA for these genes. This effect is the counter to that expected from n-3 PUFAs where in human studies, decreased expression of *CD36*, *CD14* and *PDK4* mRNA is observed in peripheral blood mononuclear cells (PBMCs) isolated after supplementation [13]. It is possible that the low-calorie diet impacted on the DNA methylation to a greater extent than the n-3 PUFAs, which only attenuated the decrease in methylation of *CD36* once adjusted for baseline body weight [84].

There are relatively few studies that have investigated the effect of n-3 PUFA supplementation on DNA methylation in adults, and of these many have confounding factors. Addressing the confounding factors of this study [84], the participants were asked not to consume seafood and to follow a detailed diet plan during the intervention period to minimise variability. However, the Spanish diet is high in shellfish/fish, on average 88.6 g/person/day are consumed [85], and therefore, the researchers may have simply replaced the n-3 PUFAs that the participants obtained from their diets with the supplementation. Although no biochemical measurements for EPA and DHA were provided in this paper [84], earlier work from the same cohort found only a small difference in EPA and DHA after the intervention, with no data comparing baseline to endpoint [86]. Future studies should include measurement of n-3 PUFA incorporation in to the investigated cell membranes, for example, into PBMCs.

Endogenous metabolism of PUFAs occurs within a cell, the initial step in the metabolic pathway takes places in the endoplasmic reticulum where LA and alpha-linolenic acid (ALA) undergo elongation of the fatty acid carbon chain by fatty acid enlongase (ELOVL) and desaturation by insertion of double bonds by Δ6-desaturase and Δ5-desaturase [87]. The Δ6-desaturase and Δ5-desaturase enzymes, encoded by the *FADS2* and *FADS1* genes respectively, are rate-limiting steps in this metabolic pathway [88,89]. Genetic polymorphisms of these genes and others related to the PUFA metabolism can significantly alter the levels of PUFAs within the body [90] and differential methylation is likely to have a similar effect. The methylation of *FADS1* is altered by a low-calorie diet with a small interaction with n-3 PUFA supplementation [84]. When investigated alone, with a higher dose of n-3 PUFA supplementation, no significant changes in methylation were observed in *FADS1* or the elongation gene *ELOVL-2* [91]. However, the methylation of two other fatty acid metabolism genes, *FADS2* and *ELOVL-5*, increased with supplementation; with a larger number of CpG sites changing methylation in females than males and a negative relationship between the DNA methylation and the gene mRNA levels observed [91].

Future targets: inflammatory gene methylation

Modulation of inflammatory gene expression occurs through transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ). Interactions between PPARγ and fatty acids result in a decrease in cytokine expression [92]. In a cancer cell line, treatment with EPA and DHA has been shown to increase expression of PPARγ gene, *PPARG* [93]. In murine models of diabetes, expression of *PPARG* is known to be modulated by DNA methylation within its promotor [94] and differential methylation is observed in type two diabetes mellitus (T2DM) [95], however the impact of n-3 PUFAs on *PPARG* DNA methylation has not been measured. Epigenetic regulation of the PPAR coactivator 1 alpha (PCG-1α) gene, *PPARGC1A*, has been implicated in T2DM in humans [96]. The regulation of the *PPARGC1A* appears to be influenced by changes in DNA methylation and has been shown to interact with PPARγ. In men, DNA methylation of the *PPARGC1A* gene promotor is significantly increased after a high-fat overfeeding diet [97]. Whereas, in obese patients fed a low-calorie diet, the methylation of *PPARGC1A* has been shown to decrease [98].

Inflammatory diseases are often characterised with chronic activation of NFkB transcription factor and release of inflammatory cytokines [99] and therefore, an important target for changes in DNA methylation by n-3 PUFAs. Associations between dietary fats and DNA methylation in the NFkB pathway, measured using the Infinium 450 k array, were observed in the Greek pre-adolescent cross-sectional cohort [44], and therefore, may provide valuable targets within intervention studies.

As previously described, there was no change in DNA methylation being observed in cytokine DNA methylation levels with gestational supplementation [73]. However, the study used low doses of DHA and it has not been investigated in adult intervention studies or with combined EPA

and DHA supplementation. Taking TNFa as an example, EPA treatment does not affect the stability of *TNFα* mRNA in cytoplasm and therefore, reductions in *TNFα* mRNA as a result of EPA treatment is likely to be the result of decreased formation rather than degradation of the cytokine [100]. The influence of n-3 PUFAs on *TNFα* expression may be the result of alterations in epigenetic mechanism, such as DNA methylation. Epigenetic regulation plays an important role in the control of *TNFα* expression [101–103]. Specifically, *TNFα* has been shown to be hypomethylated in Crohn's disease patients [104], is related to adipogenesis [105], and that CpG methylation interacts with histone methylation to silence *TNFα* expression during endotoxin tolerance [106]. Cross-sectional studies employing FFQ have found associations between *TNFα* methylation and dietary intake of certain nutrients including fats, however, no associations with n-3 PUFAs [107,108]. As seen with *TNFα*, studies examining DNA methylation must choose the tissue of interest carefully as more variability is observed between tissues from the same individual, than within tissue between individuals in the *TNFa* promotor [109].

Implications and considerations for future research

Most of evidence for the control of gene expression by CpG methylation comes from genes with CpG islands [30]. However, not all genes have CpG islands and it is not only the DNA methylation that occurs in the promotor region near the transcription start site (TSS) that plays an important role in regulation of gene expression, with evidence suggesting that 5mC in gene bodies and non-promotor regions plays an important role [110]. In genes which do not have a CpG island, including inflammatory genes *NOS2*, *IL-2*, *IFN-γ*, *MMP13*, *IL-1β* and *TNF-α*; CpG methylation proximal to the TSS has been shown to influence transcription [106,111–115]. Careful considerations must therefore be taken when designing assays to measure the DNA methylation of candidate genes.

As we have seen, investigations of the effects of n-3 PUFAs on DNA methylation are in their infancy and as such there are some points to consider when conducting this research. Within the n-3 PUFAs literature there is not yet a consensus on the required dose in humans required to produce an anti-inflammatory effect. Many of the existing supplementation studies so far investigating the epigenetic effect of n-3 PUFAs have used low doses with only small changes observed. In a review by Calder [11], it is suggested that a dose between 1.35 and 2.7 g/day of EPA is required for the anti-inflammatory effects. It is likely that there will be a dose-response interaction between n-3 PUFA supplementation and epigenetic mechanisms, as is seen with cytokine production [116]. There is also no consensus on the time frame required for epigenetic changes to occur. The largest incorporation of n-3 PUFAs into erythrocyte membranes is within the first 4 weeks of supplementation and it is not known if the epigenetic changes occur within the same time frame. There are fewer studies that measure the incorporation into PBMCs and none that consider CpG methylation alongside incorporation.

The majority of intervention studies investigating epigenetic changes do not account for the impact of the intervention on the cell population from which the DNA was extracted. This results in multiple complications, firstly if the influence of n-3 PUFAs is specific to one cell type it may not be observed if the cells are not separated prior to analysis. Secondly, changes may be falsely observed or masked with variations in cell populations. Differentiation into the cell subtypes found within blood requires changes within the epigenetic signatures and therefore, the observed changes in percentage methylation for an individual CpG may be the result of a change in the cell population rather than a result of the intervention. Data can be normalised to account for the proportion of different cells [52,91] and this is a consideration future studies should make.

Conclusion

Associations between n-3 PUFAs and differential DNA methylation have been demonstrated, with further evidence of n-3 PUFA impact on DNA methylation seen in supplementation interventions. A more targeted approach to the effects of n-3 PUFAs, and use of well-controlled supplementation studies, both *in vitro* and *in vivo*, could elucidate a mechanism through which n-3 PUFAs alter the DNA methylation within the inflammatory landscape.

Disclosure statement

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