The impact of exercise interventions and omega-3 polyunsaturated fatty acid supplementation on DNA methylation and gene expression.

by

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Thesis summary

Epigenetics is a rapidly developing field of study which investigates chemical modifications to the genome, independent of the DNA sequence, which regulate gene expression profiles. The most commonly studied epigenetic modification, DNA methylation, has been demonstrated to be influenced by lifestyle factors including diet and exercise. The modulation of DNA methylation by lifestyle factors is one potential mechanism for the reduction in disease risk induced by a healthy lifestyle. This thesis aimed to identify the impact of exercise on DNA methylation and mRNA expression and determine whether fatty acid supplementation may modulate this response.

Custom assays were developed and validated (**Chapter 4**) to assess the DNA methylation of peroxisome proliferative activated receptor gamma coactivator 1 alpha (*PPARGC1A*), interleukin 6 (*IL6*) and tumor necrosis factor alpha (*TNF*) at specific cytosine bases in genomic locations previously identified to be biologically relevant. Assays to investigate the mRNA expression of *PPARGC1A*, *IL6, TNF* and the DNA methyltransferases (*DNMT*) were validated to ensure accurate results.

In **Chapter 5**, *DNMT* mRNA expression decreased following an acute bout of exercise to volitional fatigue; whereas, no changes in DNA methylation were identified as a result of exercise or supplementation of omega-3 polyunsaturated fatty acids (n-3 PUFAs). However, an interaction was determined between exercise and n-3 PUFA supplementation for the DNA methylation of a single *IL6* CpG site. Following exercise, decreased DNA methylation and increased mRNA expression of *IL6* was detected after n-3 PUFA supplementation compared to the trial before supplementation. *IL6* methylation was correlated to the n-3 PUFA content in whole blood following supplementation suggesting increased n-3 PUFA content following supplementation may prime the cells for future exercise stimuli.

Chapter 6 sought to investigate whether acute exercise of an increased duration would modulate DNA methylation profiles and adopted a double-blind randomised repeated measures design to try and confirm the interaction between exercise and n-3 PUFA supplementation. Following a one-hour cycling bout, consisting of 45 mins cycling at 70% of $\dot{V}O_2$ followed by a 15 min time trial, we determined global

hypomethylation, a reduction in *PPARGC1A* DNA methylation and increased mRNA expression of PPARGC1A. These methylation changes were associated with a similar reduction in *DNMT* expression as reported in **Chapter 5**. In line with the positive correlations between whole blood n-3 PUFA content in the previous chapter, an increase in *IL6* methylation was determined following n-3 PUFA supplementation compared to the impact of supplementation with extra virgin olive oil; however, this relationship was not further modulated by exercise.

The focus of **Chapter 7** was the impact of acute resistance exercise on DNA methylation profiles and whether resistance training and fatty acid supplementation could modulate the epigenetic response. Acute resistance exercise was sufficient to increase DNA methylation of *PPARGC1A* and *IL6, and decrease TNF* DNA methylation in both leukocytes and skeletal muscle. However, neither resistance training nor fatty acid supplementation modulated this response. The magnitude of modulated DNA methylation of the cytokines *IL6* and *TNF* was greater in skeletal muscle than it was in leukocytes and the mRNA expression of these cytokines increased as a result of acute resistance exercise in skeletal muscle but not leukocytes suggesting tissue-specificity in the inflammatory response to exercise. The resistance exercise-induced methylation of an alternative promoter of the *PPARGC1A*, shown for the first time, suggests changes in DNA methylation may be critical for exercise-induced expression of transcript variants. In accordance with the impact of aerobic exercise (**Chapters 5 and 6)**, resistance exercise was sufficient to reduce the mRNA expression of *DNMT3a* and *DNMT3b*.

The data in this thesis indicates acute exercise can alter DNA methylation profiles; whereas, fatty acid supplementation has a limited impact on DNA methylation. Aerobic and resistance exercise was sufficient to alter DNA methylation in leukocytes; however, a more extensive response was determined in skeletal muscle following resistance exercise. Acute exercise, independent of mode, was sufficient to reduce the mRNA expression of DNMTs; whereas, resistance exercise training did not alter DNA methylation or mRNA expression of candidate genes. Despite the novel findings presented in this thesis, a number of fundamental questions remain to fully understand the epigenetic response to exercise and nutritional interventions before they can be used to target the aberrant methylation profiles.

Acknowledgements

Firstly, I would like to thank you to all the participants who gave up their time (and tissue) to enable me to complete the research included in this thesis. This research would not have been possible without your commitment and willingness. I am also thankful to Sarabjit and Martin for providing me with this opportunity to undertake this doctoral research and for the support and assistance they provided throughout. Thank you to all the staff and students within SSEHS who assisted me throughout my research. A special mention to Beth and Lynsey - I couldn't have asked for a better team to be part of, it was a privilege to work alongside both of you. I am also grateful for the help and advice from my 'work Mum and Dad' Liz and Nick!

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It is difficult to put into words what family means to me, but what I can say is that I am thankful for all the love and support all of you have given me. I can firmly say that I would not be where I am today without all of you. Dad and Morven, thank you for supporting me and encouraging me throughout not only my (long) University career but life in general, I am forever indebted to you. To my sister, Claire, thank you for all your love and support, although we have never lived further away, I feel as though we have become closer during my time in Loughborough. Finally, thank you to Gemma for everything, you have been there for me whenever I have needed you. Your love and support are the world to me.

Dedication:

I dedicate this thesis to my mum, you encouraged me to believe in myself and made me the man I am today. I miss you every day.

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List of abbreviations

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AA	Arachidonic acid
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
bcDNA	bisulfite converted DNA
BMI	Body mass index
bp	Base pairs
cDNA	Complementary DNA
CI	Confidence interval
CK	Creatine kinase
CMJ	Countermovement jump
COPD	Chronic obstructive pulmonary disease
CpG	Cytosine-phosphate-Guanine
Ct	Cycle threshold
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DMRs	differentially methylated regions
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1 (gene)
DNMT3a	DNA methyltransferase 3 alpha (gene)
DNMT3b	DNA methyltransferase 3 beta (gene)
DPA	Docosapentaenoic acid
E	Efficiency
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENCODE	Encyclopedia of DNA Elements
EPA	Eicosapentaenoic acid
EVOO	Extra virgin olive oil
EWAS	Epigenome wide associations studies
FA	Fatty acid
FAME	Fatty acid methyl ester
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase (gene)

gDNA	genomic DNA
HPLC	High-performance liquid chromatography
IL-6	Interleukin-6 (protein)
IL6	Interleukin-6 (gene)
LA	Linoleic acid
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LDH	Lactate dehydrogenase
LINE-1	Long interspersed nucleotide element-1
LUMA	Luminometric methylation assay
Mb	Myoglobin
MBP	Methyl-binding proteins
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
MVC	Maximal voluntary contraction
n-3 PUFA	omega-3 polyunsaturated fatty acids
n-6 PUFA	omega-6 polyunsaturated fatty acid
ncRNA	non-coding ribonucleic acid
PBMC	Peripheral blood mononuclear cells
PC	Protein carbonyls
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferative activated receptor gamma coactivator 1 alpha (protein)
Post-ex	Immediately post-exercise
Post-ex+1hr	One-hour post-exercise
Post-ex+3hr	Three-hours post-exercise
Post-ex+48hr	48 hours post-exercise
PPARGC1A	peroxisome proliferative activated receptor gamma coactivator 1 alpha (gene)
***PPARGC1A* ALT**	Alternative promoter of *PPARGC1A*
Pre-ex	Pre-exercise
PTM	post-translational modification
qPCR	quantitative polymerase chain reaction
R^2	Coefficient of determination
RM-ANOVA	repeated measures
RPE	Rating of perceived exertion
RT-qPCR	Reverse transcription of RNA to cDNA followed by qPCR
SAH	S-adenosylhomocysteine

SAM	S-adenosylmethionine
SFA	Saturated fatty acid
SNP	Single nucleotide polymorphism
TET	Ten-eleven translocation methylcytosine dioxygenase
TNF	Tumor necrosis factor (gene)
TNF-α	Tumor necrosis factor (protein)
TSS	Transcription start site
TT	Time trial
VAS	Visual analogue scale
$\dot{V}O_{2peak}$	Peak oxygen uptake
$\dot{V}O_{2max}$	Maximal oxygen uptake
WBC	White blood cell

Chapter 1 - Introduction

1.0 General Introduction

For many years it was hoped that the sequencing of the human genome would identify the unknown cause of various complex diseases. Despite being one of the most notable advances in genetics, for every question the sequence of the genome answers, more questions arise. One of the critical questions that the human genome project failed to answer is how genetically identical individuals can present vastly different phenotypes. The rapidly expanding field of epigenetics may explain the process from the exposure to risk factors to the development of diseases. Epigenetics focusses on the impact of lifestyle (environmental stimuli) on chemical modifications to DNA which impact the expression of genes and subsequently impact human health. This section provides an overview of genetic and epigenetic factors and how they can influence mRNA expression.

1.1 Genetics

Deoxyribonucleic acid (DNA) is the basic building block of life and consists of nucleotides (a phosphate molecule, a sugar molecule and one of four nitrogenous bases). Hydrogen bonds form between complement nitrogenous bases (adenine binds to thymine, and guanine binds to cytosine) on opposing strands to create a double helix structure (Watson and Crick 1953). The completion of the human genome project identified the genome to consist of ~3 billion nucleotides at a total length of ~2 metres; therefore, the genome is required to be packaged into chromatin structures to fit inside the nucleus (Annunziato 2008). DNA wraps tightly around histone proteins to form a nucleosome which is further condensed to form chromatin fibres and eventually chromosomes (Olins and Olins 2003).

The human genome encodes ~21000 protein-coding genes which undergo transcription into messenger RNA (mRNA) and is exported out of the nucleus where it can be translated into protein (Figure 1.1; Clancey and Brown 2008). Only ~1% of the human genome encodes protein-coding genes. Initially, the remaining proportion of the genome was considered ‘junk DNA’ because it was thought to be non-functional (Ohno 1972). The Encyclopedia of DNA Elements (ENCODE) project changed this view, indicating that a significant proportion (some estimates of up to 80%) of the ‘junk’ DNA is functional (Encode Consortium 2012). These non-coding DNA regions control

the expression of protein-coding genes via regulatory elements including non-coding RNA and regulatory regions, such as promoters, enhancers and silencers (Encode Consortium 2012; Maston, Evans, and Green 2006).

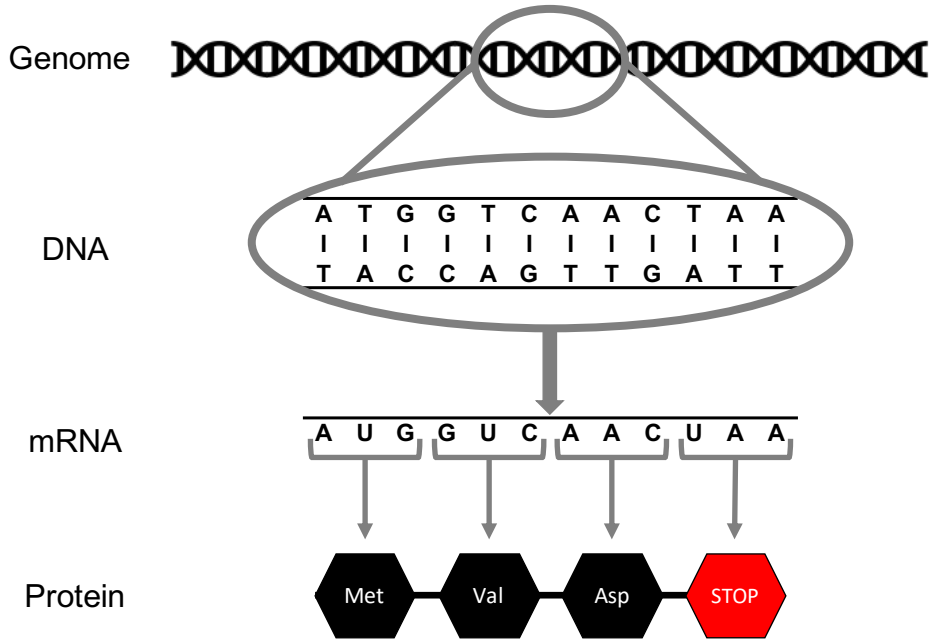

Figure 1.1- *The transcription of DNA into mRNA followed by the translation into protein.*

The sequence of any two human genomes are 99.9% identical; the variation in the remaining 0.1% produces the phenotypic differences between individuals (Kruglyak and Nickerson 2001; Shastry 2009). The most common type of sequence variant, a single nucleotide polymorphism (SNP), occurs when one nucleotide is substituted with another (Brookes 1999; Kruglyak and Nickerson 2001). SNPs occur throughout the genome in protein-coding exons, non-coding intronic regions of genes and intergenic sequences and can influence the susceptibility towards a range of diseases among other phenotypes (Brookes 1999; Shastry 2009). Exonic SNPs can exert substantial phenotypic consequences by physically impacting the biochemical properties and stability of proteins. SNPs located in introns and intergenic sequences do not alter the amino acid sequence of proteins; however, they can impact gene function via the disruption of regulatory elements and modifying splicing patterns (Baralle and Baralle 2005; Shastry 2009).

The sequence of the genome is important in determining phenotypes; however, gene-environment interactions also influence phenotypes (Ottman 1996). Twin studies have shown that genetically identical monozygotic twins can be phenotypically diverse. Phenotypic differences between monozygotic twins can range from minor anthropometric differences to susceptibility and development of disease (Fraga et al. 2005; Wong, Gottesman, and Petronis 2005). Discordance between identical genotypes and phenotypes indicates that a further layer of genomic regulation, which can be modified by the environment, exists.

1.2 Epigenetics

First coined by Conrad Waddington in 1942 (Waddington 2012), epigenetics translates to ‘above genetics’ and examines chemical modifications to DNA independent of changes to the genomic sequence (Kanherkar, Bhatia-Dey, and Csoka 2014). Every cell within an organism contains the same genetic code; however, during development the epigenome functions to create unique gene expression patterns in different cell types and create phenotypically diverse cells (Rivera and Ren 2013). Recent research has shown the epigenome is modified in response to both internal (intracellular and extracellular) and external environmental stimuli (Figure 1.2; Grazioli et al. 2017; Kanherkar et al. 2014; Martin and Fry 2018). While modifications to the epigenome are required for normal healthy physiology and to adapt to a changing environment, dysregulation of epigenetic processes can lead to the development of disease (Baylin and Jones 2016; Egger et al. 2004; Grazioli et al. 2017). The three main epigenetic mechanisms, histone modifications, non-coding RNAs and DNA methylation, are outlined below.

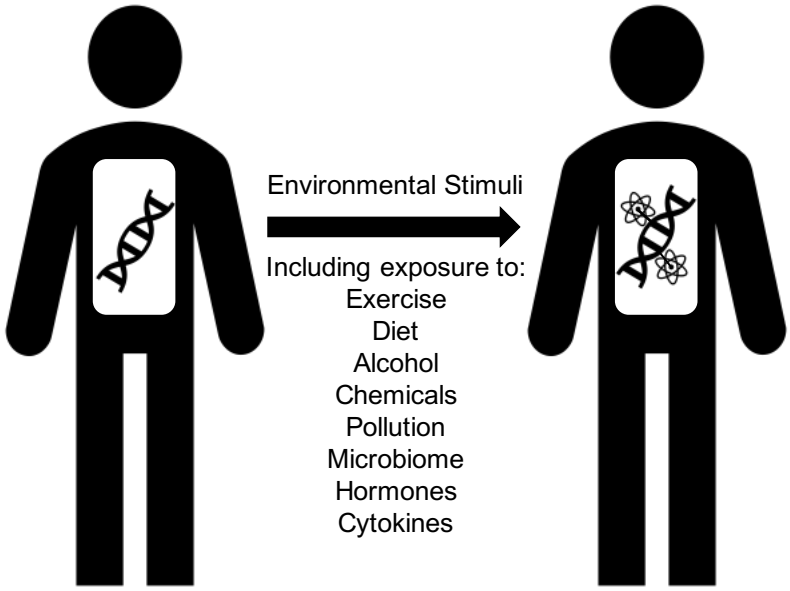

Figure 1.2 - *Examples of stimuli which can lead to epigenetic modifications.*

1.2.1 Histone modifications

The negatively charged phosphate backbone of DNA tightly binds around a complex of positively charged histone proteins (two of each histone proteins H2A, H2b, H3 and H4) to form a nucleosome (McGhee and Felsenfeld 1980). Histone proteins contain an exposed N-terminal tail that can undergo reversible post-translational modification (PTM) to make chromatin more or less accessible to RNA polymerase and transcriptional machinery (Dong and Weng 2013). The impact of PTMs on transcriptional activity depends on the type of modification (the most commonly studied histone PTMs are acetylation and methylation) and which amino acid residues of the histone tails are affected (Alaskhar Alhamwe et al. 2018).

First reported in 1964, the acetylation of lysine residues on histone tails is a process which can prevent the transcription of DNA (Allfrey, Faulkner, and Mirsky 1964). The addition of an acetyl group by histone acetyltransferases neutralises the charge of lysine making the interaction between the histones and DNA weaker. The weaker interaction between histones and DNA is associated with a more transcriptionally active chromatin state (Alaskhar Alhamwe et al. 2018). Conversely, the removal of an acetyl group, by histone deacetylases, restores the positive charge and increases the attraction between histones and DNA leading to transcriptionally inactive chromatin (Alaskhar Alhamwe et al. 2018; Dong and Weng 2013).

Unlike acetylation, the methylation of amino acid residues does not influence the ionic charge of histones (Alaskhar Alhamwe et al. 2018). The addition of methyl groups influences the transcriptional state of chromatin through the recruitment of other effector proteins (Taverna et al. 2007). Methylation occurs on either arginine residues, which can be mono- or di-methylated; or lysine residues, which can be mono-, di- or tri-methylated (Jiang, Agrawal, and Boosani 2018). Histone methylation can either result in increased or decreased transcriptional activity dependent on the specific residue that is affected. For example, tri-methylation of the lysine residue at position 4 on H3 (H3K4me3) is associated with gene transcription, whereas, tri-methylation of the lysine at position 27 of H3 (H3K27me3) is associated with gene silencing (Alaskhar Alhamwe et al. 2018).

1.2.2 Non-coding RNAs

As previously mentioned, less than 1% of the genome encodes for functional proteins. There are thousands of transcripts termed non-coding RNA (ncRNA) which despite not being translated into proteins function in an epigenetic capacity to regulate transcriptional processes (Peschansky and Wahlestedt 2014). There are many different subcategories of ncRNAs which are generally divided into two categories, short ncRNA and long ncRNA.

While there are several different classes of short ncRNA, the most commonly studied are microRNA (miRNA). MiRNAs, first discovered in 1993 (Lee, Feinbaum, and Ambros 1993), are ~22 nucleotides in length and function to alter the translation of mRNA into protein. In humans, over 2000 miRNAs have been identified (Hammond 2015). The ability of a single miRNA to target several genomic sequences allows the majority of genes to be regulated by miRNAs (Zhang and Wang 2017). Typically miRNAs bind to the 3’ untranslated region of mRNA in the cytoplasm, via base pair complementarity, marking the transcript for degradation and subsequently repression expression (Peschansky and Wahlestedt 2014). Recent evidence has identified the presence of miRNA in the nucleus of cells (Roberts 2014) and when bound to enhancers miRNA can induce transcriptional activation by inducing histone modifications (Xiao et al. 2017).

Long ncRNAs, defined as ncRNA 200 bp or longer, are the least studied ncRNA (Frías-Lasserre and Villagra 2017). Despite the lack of research on the function of long ncRNA, over 15,000 long ncRNA genes have been identified (Morlando and Fatica 2018). Long ncRNAs are involved in guiding epigenetic regulators (acetyltransferases, methyltransferases, etc.) to specific genomic locations and controlling chromatin folding to allow communication between enhancers and promoters (Frías-Lasserre and Villagra 2017; Morlando and Fatica 2018). One of the most commonly studied long ncRNA *Xist* functions to inactivate one of the X chromosomes in females (Frías-Lasserre and Villagra 2017). *Xist* transcripts coat the X chromosome, marking it to be silenced, and initiates histone modifications associated with transcriptionally inactive chromatin including acetylation and increased *H3K27me3* (Ponting, Oliver, and Reik 2009).

1.2.3 DNA methylation

DNA methylation involves the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the 5th carbon of a cytosine in a reaction catalysed by DNA methyltransferases (DNMTs) to form 5-methylcytosine (5mC; Figure 1.3) (Moore, Le, and Fan 2013). Methylation usually occurs at CpG dinucleotides (a cytosine followed by guanine); however, CpG dinucleotides only occur at 20% of the expected frequency, and ~80% of these CpG sites are methylated (Ehrlich et al. 1982) indicating some selection pressure on these nucleotides. Despite an underrepresentation of CpG sites, there are regions within the genome with dense clusters of unmethylated CpG dinucleotides which are referred to as CpG islands (Gardiner-Garden and Frommer 1987). These CpG islands overlap with the promoter region of ~60-70% of human genes (Saxonov, Berg, and Brutlag 2006) and function to restrict gene expression (Illingworth and Bird 2009).

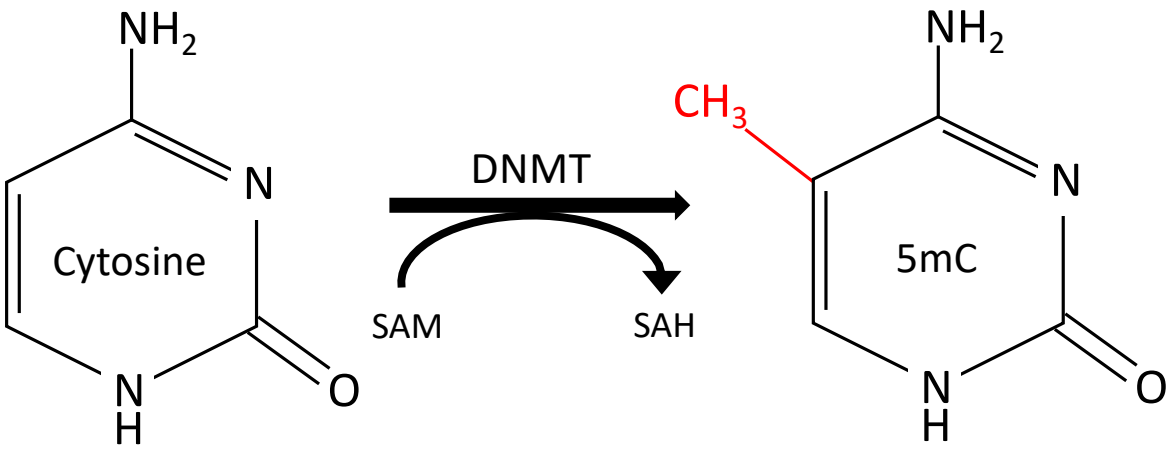

Figure 1.3 - *The DNA methylation process. DNMTs catalyse the addition of a methyl group from the methyl donor S-adenosylmethionine (SAM), which is converted into S-adenosylhomocysteine (SAH), onto a cytosine forming 5-methylcytosine (5mC).*

DNA methylation was first demonstrated to influence gene regulation in the 1980s (Compere and Palmiter 1981). In promoter sequences, and the first exon (Brenet et al. 2011), DNA methylation is negatively associated with gene expression (Jones 2012). The presence of methyl groups (i.e. methylated DNA) co-attracts methyl-binding-proteins (MBP), which blocks transcription factors and RNA polymerase binding to the region surrounding the transcription start site (TSS) inducing an inactive chromatin state (Figure 1.4) (Bird and Wolffe 1999; Bogdanović and Veenstra 2009). While strong evidence exists for the impact of promoter methylation, the association between gene body methylation and gene expression is not as clear. Methylation of gene body CpG sites typically does not induce transcriptional silencing, suggesting that CpG methylation blocks transcription initiation but not transcription elongation (Jones 2012). Previously a positive correlation has been reported between gene body methylation and gene expression (Aran et al. 2011), whereas, gene body methylation has also been suggested as a mechanism controlling exon splicing (Figure 1.4) (Chodavarapu et al. 2010; Shukla et al. 2011).

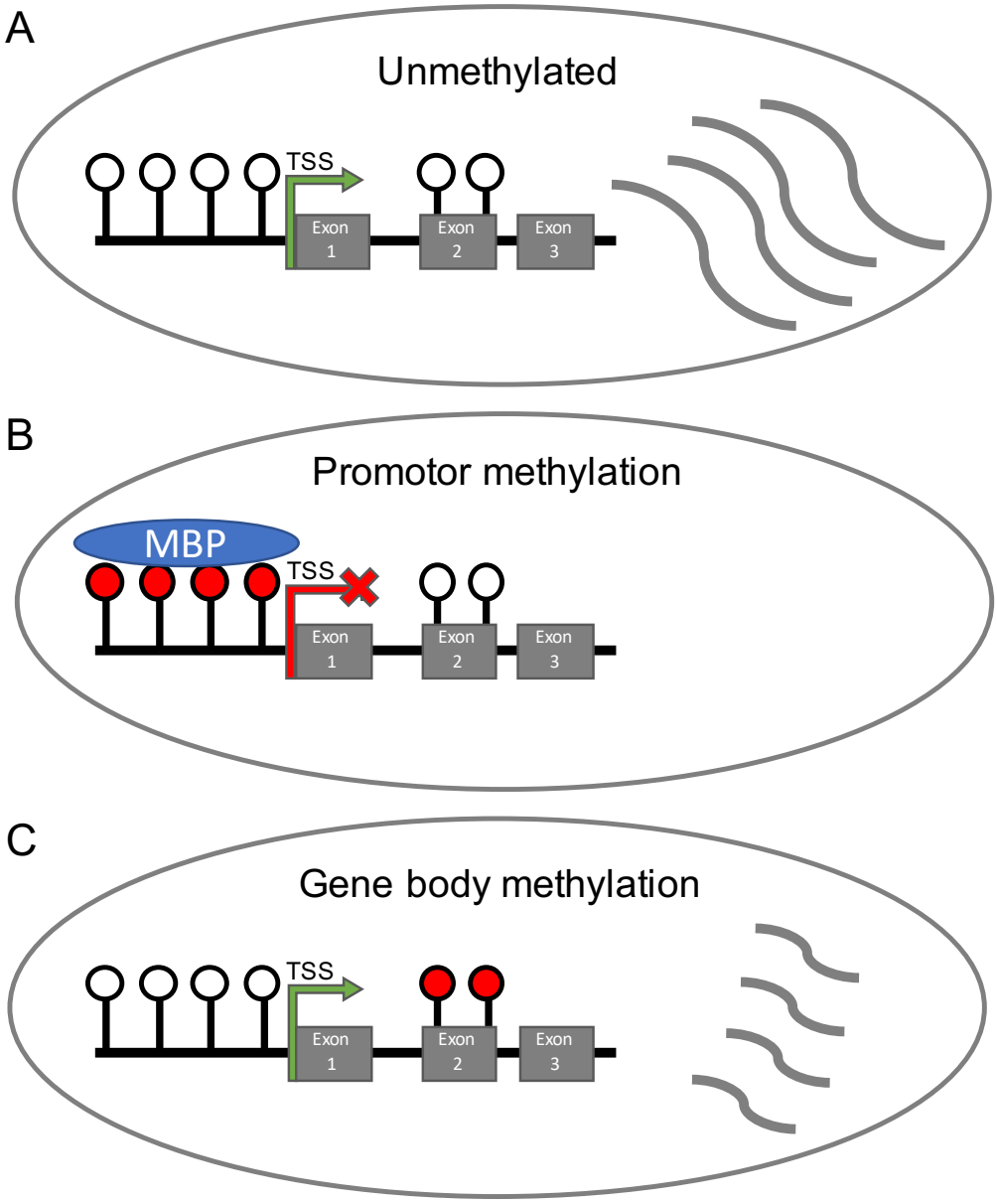

Figure 1.4 - *The impact of different methylation states on gene transcription. Red circles indicate methylated CpG dinucleotides; Clear circles indicate unmethylated CpG dinucleotides. A) Lack of methylation in promoter or gene body leads to the transcription of full-length mRNA transcripts. B) Methylated promoter leads to the binding of methyl-binding proteins (MBP) blocking access to the transcription start site (TSS) preventing transcription. C) Unmethylated promoter leads the initiation of transcription; however, gene body methylation leads to alternative splicing and the formation of a shorter transcript.*

1.2.3.1 DNA methyltransferases

The DNMT family of enzymes, consisting of *DNMT1*, *DNMT3A* and *DNMT3B*, regulate the DNA methylation process (Moore et al. 2013). DNMT1, the most abundant DNMT in adult humans, is known as the maintenance DNMT and preferentially targets hemimethylated sequences to maintain methylation during DNA replication (Pradhan et al. 1999). *DNMT3a* and *DNMT3b* are required for *de novo* methylation and have equal affinity for hemimethylated and non-methylated DNA allowing them to methylate previously unmethylated sequences (Okano et al. 1999). Despite the classification as maintenance and de novo DNMTs, there is a degree of functional overlap between the DNMTs (Chen et al. 2003). DNMT3L, a third member of the DNMT3 exists; however, it lacks the ability to methylate DNA. Despite a lack of methylase activity, DNMT3L can increase the activity of *DNMT3a* and *DNMT3b* by increasing their ability to bind to the methyl donor SAM (Kareta et al. 2006). The expression of miRNAs have been demonstrated to be a key regulator of DNMT mRNA expression indicating further overlap between epigenetic mechanisms (Duursma et al. 2008; Fabbri et al. 2007; Garzon et al. 2009; Xu et al. 2017).

1.2.3.2 DNA demethylation

Passive DNA demethylation can occur through the inhibition of DNMT enzymes which induces hypomethylation by preventing the maintenance of methylation following cell replication (Chen et al. 2003). The lack of an enzyme capable of directly cleaving methyl groups off DNA indicates that other mechanisms must control the active DNA demethylation process (Jones 2012). The most commonly studied mechanism of active demethylation is via the ten-eleven translocation methylcytosine dioxygenase (TET) family of enzymes which can oxidise 5mC into 5-hydroxymethylcytosine (5hmC). TET can further oxidise 5-hmC into 5-formylcytosine and then 5-carboxycytosine. Thymine DNA glycosylase can then cleave the by-products of TET oxidation off DNA via the base-excision repair pathway to form cytosine (Figure 1.5) (Moore et al. 2013). DNMTs have been implicated as having a potential role in the active demethylation process. In oxidative conditions, DNMTs can switch from being DNA methylases to converting 5-hmC into cytosine (Chen, Wang, and Shen 2012), whereas in the presence of high intracellular calcium concentrations, DNMTs can convert 5mC into cytosine (Chen, Wang, and Shen 2013).

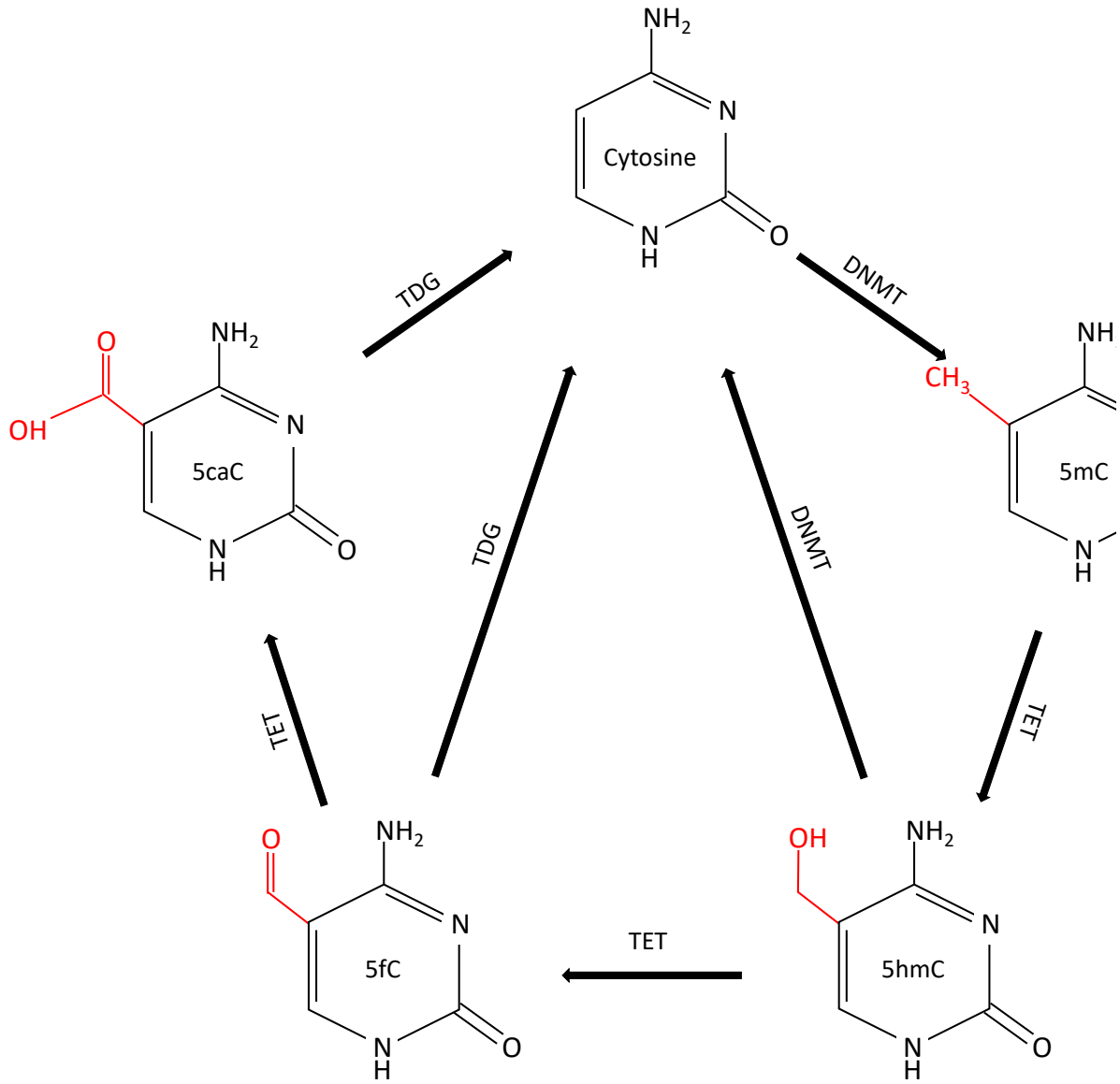

Figure 1.5 – *Active DNA demethylation cycle. Modifications between steps are shown in red. DNMT, DNA methyltransferase; TET, Ten-eleven translocation methylcytosine dioxygenase; TDG, Thymine DNA glycosylase; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxycytosine.*

Chapter 2 - Literature review

2.1 Introduction

As described in Chapter 1, there are overlapping functions between the different epigenetic modifications to regulate mRNA expression of the underlying genomic sequence. Each of these epigenetic modifications are critical during development (Rivera and Ren 2013) and throughout life in response to both internal and external stimuli and are essential for normal healthy physiology and to adapt to a changing environment (Alegría-Torres, Baccarelli, and Bollati 2011; Grazioli et al. 2017; Kanherkar et al. 2014; Martin and Fry 2018). While the optimal approach would be to investigate each of the epigenetic modifications (histone modifications, non-coding RNAs and DNA methylation) to gain an insight on the overall epigenetic impact, this approach is not always feasible because of the high analysis costs associated with epigenetic analysis. Studies typically focus on one of the epigenetic modifications, most commonly DNA methylation, to investigate the epigenetic consequence of environmental stimuli on health. The dysregulation of the methylome has been associated with the development of diseases including global hypomethylation and various forms of cancer (Friso et al. 2013), ischemic heart disease and stroke (Baccarelli et al. 2010); whereas, global hypermethylation is associated with type 2 diabetes (Simar et al. 2014).

The plasticity of the epigenome is highlighted by the influence of various environmental stimuli on alterations of DNA methylation. An emerging area of research is the impact of lifestyle interventions on the methylome. The term lifestyle is used to describe the usual way of life of an individual and includes a range of different factors including, but not limited to, exercise, diet, smoking status and sleeping patterns (Alegría-Torres et al. 2011). These lifestyle factors are intertwined with health and have been the subject of intense research, partly motivated by increasing rates of chronic diseases in industrialised societies. While the sequence of the genome is critical determining health, there is the potential for these lifestyle factors to induce significant remodelling of the methylome (Alegría-Torres et al. 2011; Choi and Friso 2010; Grazioli et al. 2017). The focus of this thesis was the association between lifestyle interventions (exercise interventions and fatty acid (FA) supplementation) and DNA methylation. The following section reviews the current understanding of the impact of exercise interventions, and supplementation of dietary fatty acids on DNA methylation.

2.2 Exercise

Physical inactivity is among the top 10 risk factors for all diseases and is reported to be responsible for 9% of all deaths worldwide (Lee et al. 2012). The American College of Sports Medicine recommends that most adults (18–65 years) engage not only in moderate-intensity aerobic training (≥30 $min \cdot d^{-1}$ on ≥5 $d \cdot wk.^{-1}$ for a total of ≥150 $min \cdot wk.^{-1}$) but also resistance exercise for each of the major muscle groups (Garber et al. 2011). Several studies have reported that both aerobic and resistance exercise can improve risk factors related to different diseases (Ceci et al. 2014) and reduce the incidence of lifestyle diseases including cardiovascular disease, type 2 diabetes and chronic obstructive pulmonary disease (COPD) (Mador et al. 2004; Matelot et al. 2016; Sigal et al. 2007). Although the benefits of exercise are well reported, a third of the world's population fails to achieve the prescribed exercise recommendations (World health organisation 2010).

The mechanisms underlying the exercise-induced health benefits remain to be fully elucidated. One suggested mechanism is via the extensive transcriptional changes induced by both acute exercise and exercise training for the adaptation process to occur (Coffey and Hawley 2007; Egan and Zierath 2013; Gjevestad et al. 2017; Gjevestad, Holven, and Ulven 2015; Zierath and Hawley 2004). It is thought that exercise-induced alterations to the epigenome are a contributing factor underlying this change in mRNA expression as a result of exercise. Over the past decade, an increasing number of studies have investigated the impact of different exercise interventions on DNA methylation and the subsequent impact of transcriptional control in a range of different tissues including leukocytes, skeletal muscle and adipose tissue. The following section covers the impact of aerobic and resistance exercise interventions on DNA methylation patterns. Articles were included in this review if they investigated the interaction between DNA methylation and either acute exercise or exercise training in humans.

2.2.1 Aerobic Training studies

The majority of exercise studies have investigated the impact of aerobic exercise on the DNA methylation. Studies investigating the impact of global DNA methylation have so far failed to determine any change in global DNA methylation following training (Duggan et al. 2014; King-Himmelreich et al. 2016; Lindholm et al. 2015). A methodological flaw with global methylation techniques is the limited conclusions which can be made because it only provides an overview of the directionality of the changes and not an indication of the individual CpG sites which are changing (Lisanti et al. 2013). Therefore, although these global studies may have determined that there is no net change in methylation; it cannot be determined that the methylome has not undergone extensive remodelling resulting in no absolute change in methylation. While some of the studies have also used either gene-specific (King-Himmelreich et al. 2016) or genome-wide association (EWAS) (Lindholm et al. 2015) investigation of methylation of gain insight of the impact on actual CpG sites, others have not (Duggan et al. 2014).

In support of the limitation of global analysis, the genome-wide investigation of three-months of aerobic exercise training in one leg by Lindholm et al., (2015) mentioned above identified altered DNA methylation of 4,919 CpG sites (839 > 5%) in skeletal muscle suggesting extensive remodelling of the genome. The function of pathways regulated were identified to be involved with muscle structure, function, and bioenergetics, suggesting that epigenetic changes are at least associated with adaptation within the muscle. Two other EWAS of aerobic training have been conducted in skeletal muscle and have indicated a hypomethylation response induced by exercise training (Nitert et al. 2012; Rowlands et al. 2014). In a cohort of obese Polynesians with type 2 diabetes (n = 18) the impact of aerobic exercise training was investigated in half of the participants and determined a net hypomethylation with pathway analysis of the significant sites that were differentially methylated indicating functions in metabolism; cardiovascular development and function; and hematological system development and function (Rowlands et al. 2014).

In individuals with family history of diabetes, a six-month training intervention consisting of predominately aerobic based exercises (three times per week for one

hour), induced differential methylation of 134 genes (Nitert et al. 2012). The majority of the genes were hypomethylated with only 19 genes becoming hypermethylated. The hypomethylated genes included *RUNX1* and *MEF2A*, which are key transcription factors involved in exercise training adaptation (McGee et al. 2006), and *PPARGC1A* which encodes for peroxisome proliferator-activated receptor gamma, co-activator alpha (PGC-1**α**) and considered to be the master regulator of mitochondrial biogenesis (Ventura-Clapier, Garnier, and Veksler 2008). Interestingly a separate analysis of adipose tissue from the same cohort detected a global hypermethylation response and modulated methylation of 17,975 CpG sites in 7,663 genes (Rönn et al. 2013). The extensive hypermethylation indicated that the methylome of adipose tissue is remodelled following exercise training; however, the response is opposite to skeletal muscle (hypomethylation vs hypermethylation). These data provide strong evidence of the tissue-specific DNA methylation response which can occur.

Aerobic exercise training has also been demonstrated to alter the methylation profile in blood leukocytes (Denham, O'Brien, Marques, et al. 2015; Zhang et al. 2015). A four-week exercise training intervention in previously untrained healthy males (n = 26) was sufficient to alter the methylation (between 0.1% to 62.8%) in ~200,00 CpG sites. Overall the response favoured a hypomethylation response with over 120,000 sites decreasing in methylation. Interestingly the methylation of UNG, which is involved in base-excision repair during demethylation, and various miRNA genes were increased following exercise. These data provide a suggested pathway for multiple epigenetic modifications working together to elicit exercise adaptions. Six-months of regular low-intensity exercise has also been demonstrated to be sufficient to induce an altered methylation profile in leukocytes of elderly individuals (Zhang et al. 2015). Following the exercise training, 40 genes were differentially methylated in the exercise group compared to the control group. Within this study, they also used a gene-specific approach to confirm the findings of hypermethylation of NFκB2, which is known as the master gene of inflammation (Tak and Firestein 2001). The same exercise intervention as reported by Zhang et al. (2015) has previously been demonstrated to induce hypermethylation of the ASC gene, which is a mediator of inflammatory signalling (Nakajima et al. 2010). Interestingly the methylation of the gene was determined to be decreased in an older control group compared to a younger group suggesting exercise may help to recover the age-associated decline in ASC gene methylation (Nakajima

et al. 2010). Taken together, these data indicate that that long-term exercise may suppress the expression of cytokines through increased DNA methylation of genes, including *NFκB2*, related to inflammatory processes (Nakajima et al. 2010; Zhang et al. 2015).

Aside from the inflammatory response, the other main pathway identified to be regulated by exercise interventions in EWAS were metabolism and mitochondrial biogenesis. Gene-specific analysis have investigated the impact of genes related to these processes. Physical inactivity, although not an exercise intervention, in the form of nine days of bed rest has been reported to be sufficient to induce significant hypermethylation of the *PPARGC1A* promoter in the skeletal muscle of a cohort of young healthy participants (n = 20)(Alibegovic et al. 2010), the opposite to the previously reported hypomethylation reported following exercise training (Nitert et al. 2012). After completing the period of bed rest, participants then completed a four week aerobic training period which was insufficient to fully return *PPARGC1A* methylation to baseline values (Alibegovic et al. 2010). These data indicate the full relationship *PPARGC1A* methylation has with exercise (or a lack of exercise) and that even short durations of physical inactivity can cause relatively persistent modifications to the methylome.

In the study by King-Himmelreich et al. (2016) discussed above, the exercise intervention was insufficient to alter global DNA methylation in leukocytes; however, when a gene-specific approach was used the methylation of a single CpG site in the AMPKα2 gene promoter was hypermethylated. AMPK is known as a critical molecular sensor and is activated by elevated by increases in the AMP/ATP ratio as a mechanism to maintain energy homeostasis (Witczak, Sharoff, and Goodyear 2008), although well characterised in skeletal muscle in response to exercise the regulation in leukocytes is poorly studied (Quentin et al. 2011); therefore, the function of the hypermethylated AMPKα2 promoter in leukocytes is unknown.

2.2.2 Acute bouts of aerobic exercise

The previous aerobic training studies demonstrate that regular exercise provides a sufficient stimulus to exert significant changes to the methylome and alter mRNA expression patterns, potentially one of the mechanisms controlling the exercise-induced adaptation process. The impact of acute aerobic exercise on DNA methylation is only starting to be elucidated.

The genome-wide influence of exercise on DNA methylation in leukocytes was analysed in a cohort of endurance trained males (n = 8) (Robson-Ansley et al. 2014). The exercise bout consisted of a 120-minute treadmill run at 60% of $v\dot{V}O_{2max}$ interspersed with sprints at 90% of $v\dot{V}O_{2max}$ for the last 30 seconds of every 10 minutes, followed by a 5 km time trial and has previously been demonstrated to transiently induce elevated IL-6 (Walshe et al. 2010). No significant changes in methylation were detected following exercise suggesting a limited impact of acute exercise on leukocyte methylome; however, the DNA methylation of 11 genes was significantly correlated to the exercise-induced increase in plasma IL-6 concentration immediately post-exercise. The majority of the genes identified were involved in immune activities, including IRAK3 which is a key inhibitor of inflammation associated with metabolic syndrome and obesity.

Interestingly in a cohort of COPD patients (n = 10), a single training session of concurrent exercise was sufficient to decrease global methylation levels in plasma (da Silva et al. 2017). However, following eight weeks of training (3 sessions per week), there was no impact following a further acute bout of exercise. The decrease in methylation following a single bout in untrained individuals may occur as a process for the adaptation to the exercise bout whereas, consistent with the findings of Robson-Ansley et al. (2014), the lack of hypomethylation following eight-weeks of training suggests that acute exercise is not sufficient to alter the methylome of trained participants. The lack of response in trained individuals may be explained if the exercise-induced adaptations associated with the bout exercise have occurred; therefore, the methylation response may be maintained and not responsive to future bouts of exercise.

Alternatively, the inconsistent findings between pre- and post-training acute exercise in the study by da Silva et al., (2017) could be explained by a methodological flaw in the selection of plasma samples as the source of DNA for analysis. Increased cell-free DNA has been reported following exercise and muscle tissue is likely to be the source of increased DNA because of exercise-induced muscle damage (Atamaniuk et al. 2004), however, the muscle adapts to the exercise stimulus and is thereafter less susceptible to damage following subsequent bouts of the same exercise (Peake, Nosaka, and Suzuki 2005). Therefore, the lack of results following training may be explained if different tissues are contributing to the pool of cell-free DNA because of differences in methylation between tissue types.

In support of the training status based response, DNA hypomethylation was determined in a cohort of sedentary individuals following an acute bout of exercise to volitional fatigue (Barrès et al. 2012). The analysis was furthered by the investigation of promoter methylation, and mRNA expression of genes related to mitochondrial function and fuel usage in a cohort of sedentary males (n = 8). Barres and colleagues (2012), identified an exercise-intensity dependent hypomethylation of *PPARGC1A*, *TFAM, PPARδ, MEF2A* and *PDK4* following high- (80% $\dot{V}O_{2peak}$), but not, low-intensity (40% $\dot{V}O_{2peak}$) energy-expenditure matched exercise. The decreases in methylation were identified alongside an increase in the mRNA expression of *PPARGC1A*, *TFAM, PPARδ* and *PDK4*; however, no change in mRNA expression of *MEF2A* was identified despite a hypomethylation response. Previously exercise intensity has been identified as the critical limiting factor in PGC-1α activation within skeletal muscle (Egan et al. 2010); however, these results were the first to demonstrate a role of methylation in regulating the exercise-induced increase in key signals for increased mitochondrial biogenesis.

The findings of exercise-induced DNA methylation of *PPARGC1A* are of particular interest because of its role as a master regulator of mitochondrial biogenesis (Ventura-Clapier et al. 2008). Further investigation of the exercise-induced epigenetic activation of *PPARGC1A* was performed in a recent study in healthy males (n – 11) who exercised on a cycle ergometer at 50% of $\dot{V}O_{2max}$ until they expended 650 kcal (Bajpeyi et al. 2017). Skeletal muscle nucleosome occupancy surrounding the CpG site -260 bp from the TSS of *PPARGC1A* was reduced and mRNA expression

increased (Bajpeyi et al. 2017). Further when the cohort was divided into high and low responses based on the hypomethylation of the -260 CpG site, those with the largest hypomethylation response (high responders) displayed a significant increase in *PPARGC1A* mRNA expression. These results provide strong evidence for the functional role of *PPARGC1A* methylation in controlling the post-exercise response of *PPARGC1A* mRNA expression, a key molecular adaptation for mitochondrial biogenesis.

Acute exercise (120 min steady state exercise at ~50% of $\dot{V}O_{2peak}$) in a cohort of trained cyclists (n = 7) has also been investigated for effect on skeletal muscle methylation for genes related to mitochondrial biogenesis and fuel utilisation (Lane et al. 2015). Methylation of COX411 and FABP3 increased four hours post exercise independent of nutritional status (both fasted – carbohydrate depleted - and fed – normal -conditions), whereas, PPARδ only increased in methylation in the fasted trial. Although the impact of methylation changes on mRNA were unclear, these data indicate that acute exercise in trained individuals induced a hypermethylation response. When considering these three candidate gene studies together, the limited impact in trained compared to untrained individuals suggests that training status may be an important factor in determining the methylation response to acute aerobic exercise (Bajpeyi et al. 2017; Barrès et al. 2012; Lane et al. 2015).

These acute exercise studies indicated that exercise is sufficient to induce global hypomethylation in untrained individuals (Barrès et al. 2012; da Silva et al. 2017); however, there are genome-wide epigenetic consequences in exercise-trained individuals (Robson-Ansley et al. 2014; da Silva et al. 2017). Potentially the lack of extensive reprogramming of the methylome in trained individuals is because the exercise-induced adaptation process has already occurred and the exercise stimuli was insufficient to further alter methylation profiles (Robson-Ansley et al. 2014). Studies utilizing a candidate gene approach have focused on the methylation of genes related to mitochondrial biogenesis and fuel utilisation and demonstrated a hypomethylation response in skeletal muscle (Bajpeyi et al. 2017; Barrès et al. 2012; Lane et al. 2015); however, there is a lack of investigation in other tissues, including leukocytes.

2.2.3 Resistance exercise studies.

Compared to the number of studies which have assessed the impact of aerobic exercise on DNA methylation, a lack of literature exists on the impact of resistance exercise (Denham et al. 2016; Dimauro et al. 2016; Rowlands et al. 2014; Seaborne et al. 2018). The first study to investigate the impact of resistance exercise, examined the impact of a 16-week resistance training protocol on genome-wide DNA methylation profiles from skeletal muscle of Polynesian adults (n = 9) with type 2 diabetes (Rowlands et al. 2014). The resistance training protocol was sufficient to induce a global hypomethylation response; however, aerobic training elicited a greater degree of hypomethylation, potentially suggesting that the genome is less responsive to resistance training compared to aerobic training. Investigation of the molecular pathways affected indicates that networks related to cellular assembly and organisation, cellular development, tissue morphology, and cardiovascular system were the most affected by resistance exercise. In support of the resistance-exercise-induced hypomethylation reported by Rowlands et al., (2014), a 12-week explosive-type resistance training protocol in a cohort of elderly individuals (70 – 75 yrs; n = 10) was sufficient to induce an 18% decrease in global leukocyte methylation (Dimauro et al. 2016); however, the ELISA based detection utilised prevents the identification of the CpG sites which are affected.

A second study investigating the impact of resistance training on leukocyte methylation identified large-scale remodelling of the methylome following an eight-week resistance training protocol (3 sessions per week of 3 sets, 8-12 reps) in a cohort of healthy participants (n = 8) (Denham et al. 2016). In total altered DNA methylation (0.1 – 27.2%) was identified in ~57,000 CpG sites with ~28,000 displaying hypermethylation and ~29,000 displaying hypomethylation providing more evidence that resistance exercise favours a hypomethylation response of the genome (the actual overall change in methylation is unknown). Various biological pathways were identified to be differentially methylated including type 2 diabetes, calcium signalling, axon guidance, and differentially methylated growth factors which are known to be critical for the anabolic impacts on skeletal muscle including insulin, insulin growth factor 1 receptor and growth hormone-releasing hormone (GHRH). Interestingly the mRNA expression of GHRH was also increase following the resistance training. Nonetheless, these data

suggest a role of resistance training in remodelling the leukocyte methylome. Despite the widespread remodelling of the methylome following resistance exercise it must be acknowledged that a previous study by the same authors identified a greater number of CpG sites increased in methylation and by a greater magnitude following aerobic training than resistance exercise (Denham, O'Brien, Marques, et al. 2015) which is consistent with the findings in skeletal muscle (Rowlands et al. 2014).

The final study to have investigated the impact of resistance exercise is the most extensive to date (Seaborne et al. 2018). Seaborne et al., (2018) investigated the impact of an acute bout of resistance exercise, resistance loading, unloading and subsequent reloading on the skeletal muscle methylome in a cohort of previously untrained participants (n = 8). A similar number of CpG sites ~17,000 were differentially regulated across the acute, loading and unloading periods; however, following reloading a large increase in the number of differentially methylated CpG sites was identified (~27,000). Interestingly an overall hypomethylation response was detected at all time points with a similar number of sites becoming hypermethylated in all conditions (~8,000). The PI3K/AKT pathway, critical for growth and protein synthesis, was significantly enriched in all comparisons. Further analysis identified 18 CpG sites which displayed an almost identical change in methylation across all experimental conditions indicating an important role for these sites in acute and chronic adaptation to exercise. Despite a shortage of literature investigating the impact of resistance exercise on DNA methylation, remodelling of the epigenome is suggested by all of these studies, especially for genes related to skeletal muscle growth, typically resulting in a greater hypomethylation compared to hypermethylation; however, the magnitude of the response is smaller following resistance exercise compared to aerobic exercise (Denham et al. 2016; Rowlands et al. 2014).

2.2.4 Summary of DNA methylation response to exercise

Taken together the literature above clearly demonstrates that both acute and chronic, aerobic and resistance exercise is sufficient to modify the DNA methylome. Exercise, regardless of mode; intensity and duration, causes both hypomethylation and hypermethylation of specific CpG sites; however, hypomethylation is more frequently reported. One of the most commonly studied targets, PAPRGC1A, is hypomethylated following acute aerobic exercise and aerobic exercise training; however, it is unknown whether resistance exercise would alter *PPARGC1A* methylation. Despite no literature surrounding the methylation of *PPARGC1A* following resistance exercise, mRNA expression studies have shown resistance exercise can induce significant expression of *PPARGC1A* from an alternative promoter (Ruas et al. 2012; Silvennoinen et al. 2015). These data suggest a potential epigenetic response which should be investigated in future studies.

2.3 Nutrition

Nutrition is one lifestyle factor which influences health throughout life.

The Developmental Origins of Health and Disease hypothesis explains how abnormalities within the developmental environment impacts the epigenome and can transition into metabolic disease later in life (Gluckman and Hanson 2004). Maternal stress, birth weight and foetal malnutrition can all impact upon the developmental environment, and subsequently induce modifications to the epigenome which can endure throughout the lifespan and some are passed onto future generations. The consumption of certain nutrients and bioactive foods have been demonstrated to alter epigenetic profiles and subsequently alter gene expression and improve health. The majority of studies have focussed nutrients involved in the one-carbon metabolism cycle, such as folate, methionine, betaine and other B-vitamins, because of the critical role of one-carbon metabolism in the synthesis of SAM, which is the methyl donor for DNA methylation (Anderson, Sant, and Dolinoy 2012). Aside from these critical nutrients for one-carbon metabolism, other nutritional factors such as dietary FA intake, particularly omega-3 polyunsaturated fatty acids (n-3 PUFAs), can alter DNA methylation patterns.

The following section covers what FAs are, the dietary sources, the health implications and finally summarise what is currently known of the impact of FA consumption on DNA methylation. Articles were included in this review if they investigated the interaction between DNA methylation and PUFA in humans.

2.3.1 Fatty acid background

2.3.1.1 Fatty acid structure and nomenclature

Fatty acids (FAs) are naturally occurring dietary components which have essential roles in metabolism, cell signalling, structure and function (Calder 2011; Rustan and Drevon 2005). All FAs consist of a hydrocarbon chain (most commonly an even number of carbons between 12-22) with a methyl group (CH_3) at one end and a carboxyl group (COOH) at the other (Figure 2.1). The length and structure of the hydrocarbon chain results in the classification of FA based on different functional properties. FAs with no double bonds between carbon molecules in the hydrocarbon chain (i.e. saturated with hydrogen) are termed Saturated FAs (SFA), whereas, the inclusion of double bonds leads to the classification as an unsaturated FA. Unsaturated FAs can further be separated into Monounsaturated FAs (MUFA) when only one double bond exists, and Polyunsaturated FAs (PUFA) when more than one double bond is present (Ratnayake and Galli 2009).

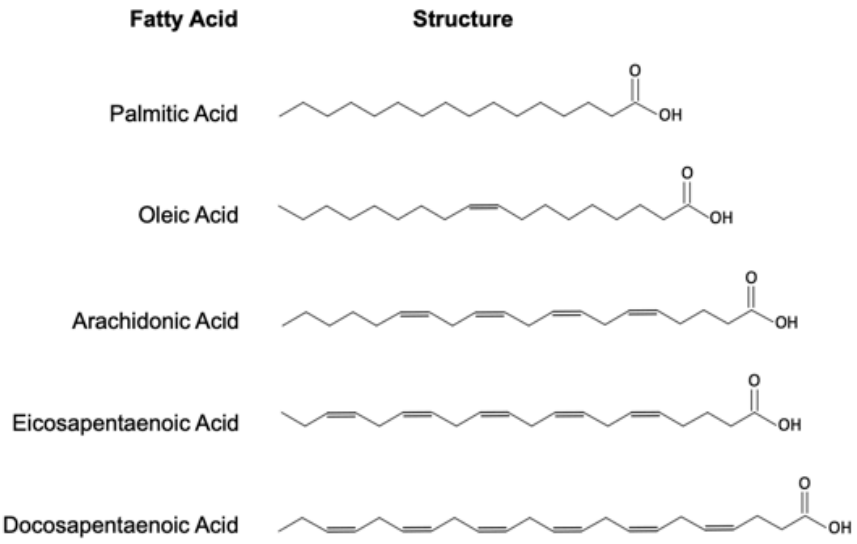

Figure 2.1 – *Chemical structure of commonly studied fatty acids.*

FAs are typically called by their common (or trivial) name, which is typically derived from the compound from which the FA was first identified. Although commonly used these names do not indicate the structure of the FA, therefore, systematic names and a shorthand notation was developed. The systematic name for FA is determined by the number of carbons and double bonds (also the orientation) in the hydrocarbon chain (Table 2.1). The location of double bonds is indicated by the carbon number it occurs at (counted from the carboxyl group). While this system works well for SFAs and MUFAs, it becomes complicated for PUFAs. Subsequently, a shorthand notation for FAs has become increasingly used. The notation identifies the number of carbons, the number of double bonds and the location of the first double bond counted from the methyl group. The location of the double bond from the methyl group is referred to as the omega number (indicated by n-X). The most common positions for these double bonds are n-3, n-6 and n-9 (Ratnayake and Galli 2009).

Table 2.1 - *Examples of the common nomenclature of commonly studied fatty acids*

Common name	Systematic name	Class	Shorthand notation
Palmitic acid	Hexadecanoic acid	SFA	16:0
Oleic acid	Cis-9-Octadecenoic acid	MUFA	18:1n-9
Arachidonic acid (AA)	All cis 5, 8, 11, 14-Eicosatetraenoic acid	n-6 PUFA	20:4n-6
Eicosapentaenoic acid (EPA)	All cis 5, 8, 11, 14, 17-Eicosapentaenoic acid	n-3 PUFA	20:5n-3
Docosahexaenoic acid (DHA)	All cis 4, 7, 10, 13, 16, 19-Docosahexaenoic acid	n-3 PUFA	22:5n-3

2.3.1.2 Dietary requirement and sources of FAs

In the early 1900's, dietary fat was only thought of as a source of calories and not considered to be important for normal physiology. Seminal work by Burr and Burr changed this view when they demonstrated diets lacking FAs caused a deficiency syndrome in rats which frequently lead to death; however, the inclusion of a few drops

of lard into the diet was sufficient for the rats to recover (Burr and Burr 1929). A further study by the same authors demonstrated the inclusion of the PUFAs linoleic acid (LA; 18:2n-6) alpha-linolenic acid (ALA; 18:3n-3) was sufficient for the animals to return to health, whereas, the addition of SFAs to the diet had no impact on the animal's condition (Burr and Burr 1930).

Unlike many of the biologically important FAs which can be synthesised within the body; LA and ALA cannot be synthesised and must be gained from an individual's diet. The requirement for the dietary intake of these essential FAs is caused by the absence of the enzymes (∆12- and ∆15-desaturase) which are required to desaturate FAs at the n-6 and n-3 position in mammals (Calder 2004). Fortunately, LA is the most abundant PUFA within the human diet and is found in a range of vegetable oils including sunflower, corn and soybean. ALA is less common within the human diet (~10% of the amount of LA) and is commonly found in walnuts and both linseed and canola oil (Russo 2009)

Despite an inability to synthesise LA and ALA, humans do possess the ability to metabolise these FAs into longer chain derivatives. LA can be metabolised into arachidonic acid (AA; 20:4n-6), whereas, ALA can be converted into eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in a series of elongation and desaturation steps (Figure 2.2). The same enzymes (Δ-5 and Δ-6 desaturases, and elongase 2 and 5) are used for the synthesis of longer chain n-3 and n-6 fatty acids. The desaturation by Δ-5 and Δ-6 desaturases are the key rate-limiting steps in the pathways leading to competition between n-3 and n-6 metabolism (Hyekyung P Cho, Nakamura, and Clarke 1999; Hyekyung P. Cho, Nakamura, and Clarke 1999). A higher affinity exists between the desaturase enzymes and n-3 PUFAs leading to the suppression of n-6 PUFA metabolism (Simopoulos 2006).

Although humans can synthesise long-chain PUFAs, the conversion process is poor, and the primary source of these PUFAs come from the diet (Plourde and Cunnane 2007). The main dietary sources of EPA and DHA come from oily fish and fish oil supplements; whereas, AA is found in meat, eggs and dairy products (Kaur, Chugh, and Gupta 2014). The original human diet was thought to contain an equal ratio of n-

6 to n-3 PUFAs; however, the present-day western diet is thought to contain a ratio around 20:1(Calder 2011; Simopoulos 2006). The increase in n-6 PUFA content of the diet is associated with a more pro-inflammatory state and increased risk of disease (Calder 2012).

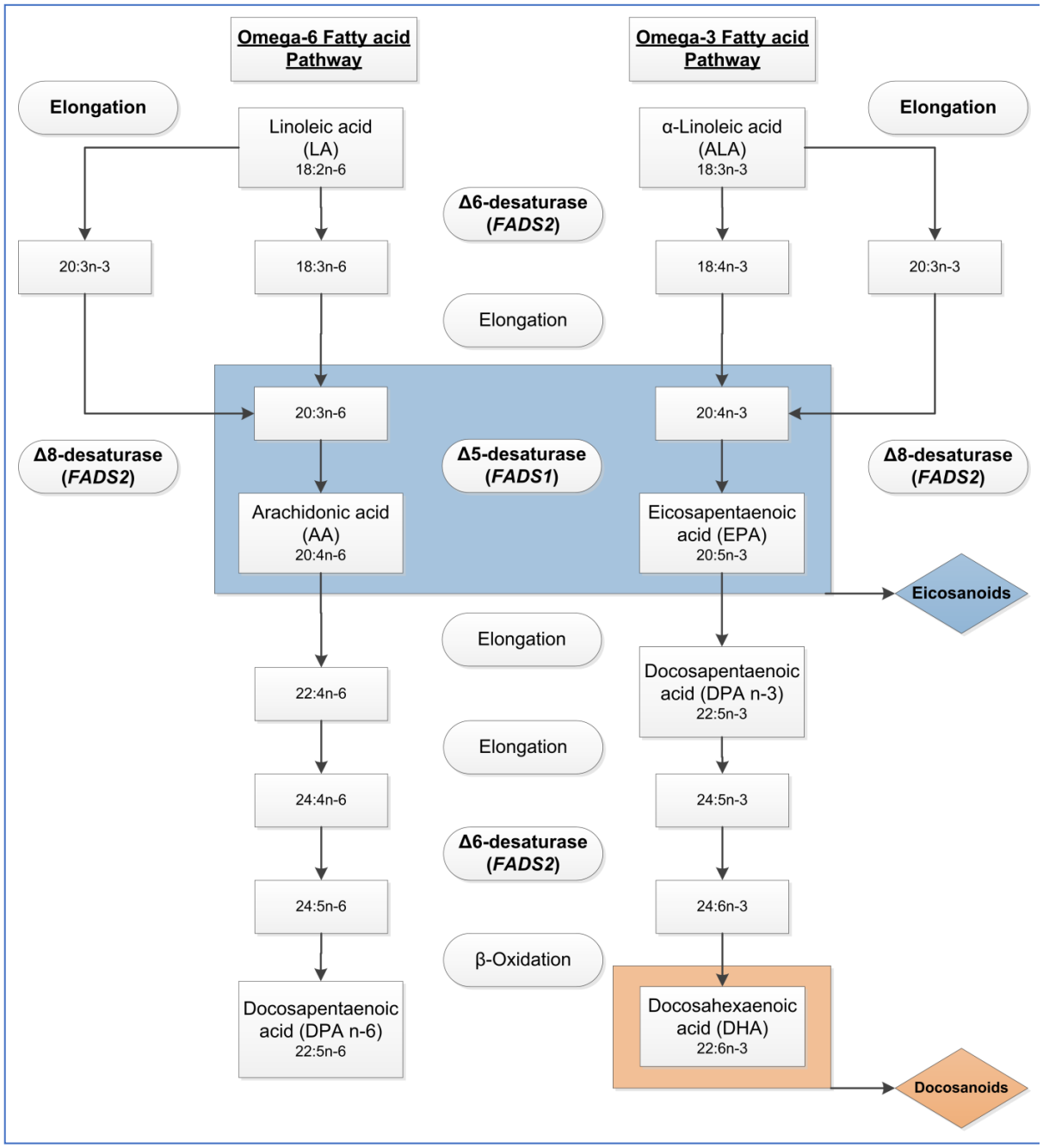

Figure 2.2 - *Figure adapted from Glaser, Heinrich and Koletzko (2010). Omega 3 and Omega 6 Polyunsaturated Fatty Acid metabolism. The Δ6-desaturase and Δ5-desaturase enzymes are encoded by the fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS2) genes. Eicosanoids and Docosanoids are key mediators and regulators of inflammation.*

2.3.2 PUFA associations with disease

Populations with diets high in n-3 PUFAs have been demonstrated to have a lower incidence of chronic disease (Simopoulos 1999). Cross-sectional epidemiology research has suggested that a high serum concentration of EPA was associated with a lower prevalence of CAD and improvements of triglyceride metabolism and HDL metabolism, and systemic inflammation (Tani et al. 2015). For example, the Mediterranean diet, which is high in MUFAs and PUFAs but low in SFA, is associated with positive health consequences including reductions in risk factors for cardiovascular disease (Estruch et al. 2018).

Studies supplementing n-3 PUFAs have sought to investigate if the dietary n-6/n-3 ratio has an impact upon disease manifestation and inflammation in humans. An increased ratio is associated with obesity (Simopoulos 2016), whereas, studies have reported that n-3 PUFAs have a beneficial effect on cardiovascular disease and chronic heart disease, but n-6 PUFA intake did not influence this relationship (Fontes et al. 2015; Mozaffarian et al. 2005). Evidence that the n-3 PUFAs, EPA and DHA, can modulate inflammation has come primarily come from *in vitro* work (Trebble et al. 2003; Verlengia et al. 2004), and animal models (Olson et al. 2013; Richard et al. 2016; Yaqoob and Calder 1995). The association in human studies is weaker because of the more complex environmental factors which need to be controlled (Itariu et al. 2012; Tartibian, Maleki, and Abbasi 2011).

While several mechanisms of action for the anti-inflammatory impact and protective health benefits of n-3 PUFA supplementation have been reported they remain to be fully elucidated (Calder 2015). Evidence suggests that n-3 PUFAs may exert health consequences via an extensive alteration to the transcriptome (Bouwens et al. 2009; Rudkowska et al. 2013; Rundblad et al. 2018); however, they may also occur as a result of altered epigenetic modifications, including DNA methylation.

2.3.3 PUFAs and DNA methylation

2.3.3.1 Cross-sectional studies

The concentrations of the n-3 PUFA EPA and n-6 PUFA AA in whole blood have been demonstrated to be positively associated with global DNA methylation in a cohort of lactating infants (n = 49) and a cohort adult male (n = 12) (de la Rocha et al. 2016). These data indicate that increased whole blood PUFA content is associated with increased global DNA methylation, whereas, global hypomethylation is associated genomic instability (Chen et al. 1998; Li et al. 2012) and cancer (Ehrlich 2009), suggesting potential health benefits. The nature of global DNA methylation prevents the identification of the specific regions of the genome which are associated with EPA and AA content.

EWAS have identified the specific genomic locations which associate with FA intake (Voisin et al. 2014) and red blood cell FA content (Aslibekyan et al. 2014). In a cohort of Yup'ik individuals with red blood cell n-3 PUFA content in either the top (n = 92) or bottom (n = 93) three deciles, DNA methylation was demonstrated to be significantly different at 21 CpG sites (Aslibekyan et al. 2014). These sites were related to genes associated with inflammation and oxidative stress suggesting an epigenetic role of n-3 PUFAs in these processes. Interestingly, positive associations were determined for the majority of these CpG sites (17/21) indicating potential global hypermethylation similar to previously reported results (de la Rocha et al. 2016).

In a cohort of Greek pre-adolescents (n = 69) estimated dietary intake of FA classes (PUFA, MUFA and SFA) from food frequency questionnaires were investigated for association with DNA methylation (Voisin et al. 2014). A total of 299 unique CpG sites were associated with PUFA/SFA, MUFA/SFA or total unsaturated FA/SFA intakes and 96 of these CpG sites were common to at least two groups. While no significant pathways were identified for MUFA intake, 34 pathways were enriched for PUFA intake including a group of pathways related to adipogenesis and another related to leptin and *IL6*. Total unsaturated FA intake enriched five pathways including a group related to *NFκB*. Unlike the previous two studies, the direction of association for the majority of CpG was negative, which would suggest potential global hypomethylation with consumption of unsaturated FAs. Potentially the difference in the direction of

association may be explained by the use of estimated intake of FAs compared to the FA content of biologically relevant tissues which directly impact the cell environment. It has been demonstrated that there are gender differences in metabolism and storage of n-3 PUFAs (Burdge, Jones, and Wootton 2002; Burdge and Wootton 2002; Lohner et al. 2013); therefore, FA intake may not accurately reflect biologically accessible FAs which would be responsible for altering DNA methylation patterns.

The endogenous metabolism of the PUFAs ALA and LA into the longer chain n-6 and n-3 PUFAs occurs through various elongation and desaturation stages (Figure 2.2). The desaturation occurs by enzymes encoded by the FADS1 and FADS2 genes and have been demonstrated to be the rate-limiting stages in the metabolic pathway (Hyekyung P Cho et al. 1999; Hyekyung P. Cho et al. 1999). Genetic polymorphisms in these genes have been demonstrated to alter the PUFA profile within the body (Chilton et al. 2014; Glaser et al. 2010). Two separate cross-sectional studies have investigated the association between FA profiles and DNA methylation levels of these critical genes for FA metabolism (Cui et al. 2016; Rahbar et al. 2018). In the first study, an inverse relationship was detected between DNA methylation of a CpG site between the proximal promoters of FADS1 and FADS2 and the AA content of prostate tumours. Further negative associations with other FA ratios which indicate the efficiency of n-6 biosynthesis and desaturation by *FADS1* were also detected (Cui et al. 2016). In two distinct cohorts of healthy adults, leukocyte and CD4+ cell DNA methylation of the *FADS2* promoter was negatively associated with circulating levels of Dihomo-γ-linolenic acid (DGLA); whereas, a positive association was detected between FADS2 promoter methylation and the DGLA / AA ratio which indicative of the efficiency of desaturation by *FADS1* (Rahbar et al. 2018). Despite differences in the direction of the associations, which may be explained by the different disease states of the cohorts, these studies suggest FA profiles may alter the DNA methylation of FADS genes and control the efficiency in which individuals convert LA into AA which may alter inflammatory processes.

Associations between inflammatory cytokines have been investigated in two cross-sectional candidate gene studies (Hermsdorff et al. 2013; Ma et al. 2016). These candidate gene studies support the findings of the previous EWAS by demonstrating

that DNA methylation of genes related to inflammatory processes may be altered by PUFA intake (Hermsdorff et al. 2013; Voisin et al. 2014) and red blood cell n-3 PUFA content (Aslibekyan et al. 2014; Ma et al. 2016). In a large cohort of 848 individuals, negative correlations were determined between red blood cell n-3 PUFA content and both *IL6* methylation and IL-6 protein concentrations (Ma et al. 2016). In a cohort of adult males (n = 40), dietary n-6 PUFA intake was determined to be one of the critical factors in determining the DNA methylation of the inflammatory cytokine *TNF* (Hermsdorff et al. 2013). *TNF* DNA methylation was negatively associated with n-6 but not n-3 PUFA intake. Hermsdorff and colleagues (2013) also report a negative association between *TNF* DNA methylation and circulating protein concentrations of TNF-α, suggesting that dietary intake of n-6 PUFAs may regulate systemic inflammatory patterns.

While the cross-sectional studies above indicate a potential epigenetic role of PUFA supplementation, particularly for genes associated with inflammation and metabolism, these studies are limited by their observational design and need to be followed up with controlled supplementation studies to determine whether PUFA supplementation is sufficient to alter DNA methylation and influence metabolic and inflammatory phenotypes.

2.3.3.2 Gestational and infant supplementation

The plasticity of the epigenome alters throughout the lifespan of an organism. During gestation, there is a period of global demethylation, followed by a remethylation phase which is critical for cell differentiation during development (Hackett and Surani 2013). Throughout gestation, the fetal epigenome is susceptible to the maternal uterine environment including exposure to smoking, famine and toxins which can alter the DNA methylation profile (Odom and Taylor 2010) of the infant. One of the critical maternal exposures has been demonstrated to be material nutrition which has led to the investigation of the impact of maternal PUFA supplementation on DNA methylation patterns in the offspring.

The impact of maternal n-3 PUFA supplementation on global DNA methylation, using LINE-1 methylation as a surrogate measure, has been investigated in two separate

studies (van Dijk et al. 2016; Lee et al. 2013). The first study supplemented mothers from 18-22 weeks gestation until birth with either 0.4 g/d of DHA (n = 131) or olive oil placebo (n = 130) and assessed DNA methylation in cord blood. No difference in LINE-1 DNA methylation was detected in the overall cohort; however, an increase in LINE-1 methylation was detected in mothers who smoked and were supplemented with DHA compared with those who smoked in the control group (Lee et al. 2013). Previously LINE-1 DNA methylation has been demonstrated to be significantly reduced in individuals who smoke (Searles Nielsen et al. 2012), and lower LINE-1 DNA methylation is associated with cancer (Ehrlich 2009). The interaction between smoking status and supplementation highlights the complex relationship between DNA methylation and the environment and suggests a potential protective mechanism of maternal DHA supplementation to negate the epigenetic impact of adverse health exposures. The second study to investigate the impact of maternal supplementation of n-3 PUFA on LINE-1 methylation, supplemented mothers from 20 weeks' gestation until birth with either 1.5 g/d of n-3 PUFA (n = 517) or vegetable oil (n = 474) and assessed DNA methylation in dried blood spots collected from the children at birth and 5 years old (van Dijk et al. 2016). No difference in LINE-1 methylation was detected either at birth of 5 years of age.

While LINE-1 methylation has been demonstrated to be a good surrogate measure of global DNA methylation (Lisanti et al. 2013), it does not indicate the epigenetic consequence at a gene-specific level. Van Dijk and colleagues (2016), conducted an EWAS in a subset of samples (n = 369) to identify differentially methylated regions (DMRs) caused by n-3 PUFA and control supplementation. In the subset analysis, a small impact of supplementation was detected with 21 DMRs identified at birth for genes related to immune function, brain function and cell membranes. The only other investigation of maternal n-3 PUFA supplementation on genome-wide methylation failed to detect any DMRs between those supplemented with 3.7 g/d of n-3 PUFA or placebo from 20 weeks of gestation to birth following the adjustment for multiple testing (Amarasekera et al. 2014). Similarly, the supplementation of nine-month-old infants with 1.6 g/d of n-3 PUFAs or 3.1 g/d of placebo (sunflower oil) for nine months failed to determine any DMRs following the adjustment for multiple testing (Lind et al. 2015).

The difference in results between these three EWAS may be explained by different sample sizes. A larger sample size was used in the study by Van Dijk et al. (2016) (n = 369) which detected a small impact of supplementation on DNA methylation compared to the studies by Amarasekera et al., (2014)(n = 70) and Lind et al., (2015)(n = 12). Therefore, a large sample size may be required to detect a difference in methylation because of the stringent adjustment for multiple testing used when using an EWAS design. The study by Lee and colleagues (2013) described above, utilised a candidate gene approach to examine the impact of maternal n-3 PUFA supplementation on methylation of inflammatory cytokines. No difference in candidate gene DNA methylation (*IFNγ, TNF, IL13, GATA3, STAT3, IL10* and *FOXP3*) was detected between supplement groups.

Unlike the cross-sectional studies above which provided consistent associations between PUFAs and DNA methylation of CpG sites related to inflammation (Aslibekyan et al. 2014; Ma et al. 2016; Voisin et al. 2014) and metabolism (Voisin et al. 2014), there is limited evidence of altered DNA methylation induced by maternal / infant supplementation of n-3 PUFAs. Potentially the lack of association following supplementation may be explained by the selection of healthy, disease-free children. Potentially supplementation of PUFAs in adults, who will have encountered more inflammatory stimuli, will be associated with modulated DNA methylation.

2.3.3.3 Adult supplementation studies

Unlike maternal supplementation studies (van Dijk et al. 2016; Lee et al. 2013), the supplementation of Alzheimer's disease patients with n-3 PUFA is sufficient to alter LINE-1 DNA methylation (Karimi et al. 2017). Six months of n-3 PUFA (1.7 g DHA and 0.6 g EPA) supplementation reduced LINE-1 DNA methylation by ~1%, whereas, no change in LINE-1 methylation was detected in the patients supplemented with an isocaloric placebo oil (1 g corn oil, including 0.6 g linoleic acid) (Karimi et al. 2017). The change in methylation following supplementation was negatively associated plasma EPA in the overall cohort (both n-3 PUFA and placebo supplemented individuals), whereas, plasma DHA was positively associated with LINE-1 methylation only in the individuals supplemented with n-3 PUFA.

Although LINE-1 hypomethylation is typically associated with adverse health outcomes, including genomic instability (Chen et al. 1998; Li et al. 2012) and cancer (Ehrlich 2009), the reduction in LINE-1 methylation in Alzheimer's patients may indicate a positive health outcome. LINE-1 DNA methylation in Alzheimer's patients is increased compared to healthy controls (Di Francesco et al. 2015); therefore, the reduction in methylation following n-3 PUFA supplementation may act to restore DNA methylation to the normal level detected in healthy individuals. The global nature of the study by Karimi et al. (2017), prevents the identification of the regions of the genome with modulated DNA methylation. In a subset of individuals, the n-3 PUFA supplementation was demonstrated to alter the expression of genes related to inflammation (Vedin et al. 2008); however, it is unknown if altered DNA methylation at these regions is responsible for the changes in gene expression. Further EWAS and candidate gene studies are required to identify the DMR following n-3 PUFA supplementation.

Overfeeding healthy adults with SFA (n = 17) or n-6 PUFA (n = 14) by 750 kcal/d for 7-weeks was sufficient to induce a genome-wide net increase in methylation. The genes affected were associated with metabolism (i.e. *PPARGC1A*) and inflammatory processes (i.e. *TNF* and *IL6*), particularly following n-6 PUFA overfeeding suggesting that FA overfeeding may alter these processes (Perfilyev et al. 2017). The increase in the number of genes affected by n-6 PUFA supplementation (1797 genes) compared to the SFA supplementation (125 genes) with limited overlap (n = 47) in genes, indicates a greater epigenetic role for n-6 PUFAs potentially explaining the metabolic differences that are induced by these two classes of FA (Rosqvist et al. 2014).

The supplementation of overweight and obese adults (n = 36) with 3 g/d of n-3 PUFA for six weeks was sufficient to alter the leukocyte DNA methylation of 308 CpG sites (Tremblay et al. 2017). Similar to the effect seen with n-6 PUFA overfeeding (Perfilyev et al. 2017), the majority of CpG sites (93%) increased in DNA methylation and pathway analysis have indicated that the genes affected are related to inflammatory and metabolic processes (Tremblay et al. 2017). The results of these two EWAS studies are in agreement with cross-sectional data suggesting that the dietary consumption of PUFAs increases the DNA methylation of genes associated with

inflammatory response and metabolism (Aslibekyan et al. 2014; de la Rocha et al. 2016)

The supplementation of kidney disease patients (n = 29) with 3.5 g/d of n-3 PUFA or 4 g/d of olive oil for 12 weeks induced gender specific alterations to DNA methylation of CpG sites in FADS1 and ELOVL5 but not FADS2 or ELOVL2. A second cohort of healthy young adults (n = 20) supplemented with 2 g/d of n-3 PUFAs for 12 weeks was also used in the study, and similar results were found. The gender-specific regulation of these CpG sites suggests an epigenetic role for the gender differences in the storage and metabolism of n-3 PUFAs previously described (Burdge et al. 2002; Burdge and Wootton 2002; Lohner et al. 2013). These results highlight the careful considerations which are required when conducting FA analysis to ensure gender differences do not confound results.

A further candidate gene study investigated the impact of n-3 PUFA supplementation in the presence of calorie restriction on the DNA methylation of genes (*CD36, FFAR3, CD14, PDK4,* and *FADS1*) which have previously been demonstrated to be downregulated in PBMCs following n-3 PUFA supplementation. Young overweight women were assigned to either the fish oil (>1.3 g/d of n-3 PUFAs) or control (<0.26 g/d of n-3 PUFAs) group for an eight-week period while following a diet plan designed to induce a calorie restriction of 30% (Amaral et al. 2014). A limited impact of n-3 PUFA supplementation on DNA methylation was detected with the main methylation impact being detected as a result of the energy restricted diet. After adjusting for baseline body mass, the methylation of the *CD36* promoter was significantly reduced by the low-calorie diet, and this reduction was attenuated by ω-3 PUFA supplementation; however, no other impact of n-3 PUFA supplementation was detected. Potentially the stimulus of the energy-restricted diet was large enough to outweigh the epigenetic impact of n-3 PUFA supplementation. These data highlight the difficulty in selecting candidate genes for DNA methylation analysis because transcriptional regulation of genes involves a wide array of different processes especially when multiple interventions are combined. 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. The study population consisted of Spanish females, and 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 habitual n-3 PUFA. Taken together these adult supplementation studies suggest a clear impact of n-3 PUFA supplementation on DNA methylation, particularly for genes involved in the inflammatory response and metabolism.

2.3.4 Summary of DNA methylation response to FAs

The literature reviewed here highlights the varied nature of the studies used to investigate the impact of FA supplementation on DNA methylation. There is limited evidence of a strong epigenetic response from gestational and infant supplementation studies, however, cross-sectional and adult supplementation studies have suggested a robust epigenetic impact of FA supplementation on genes related to inflammation and metabolism. It remains to be identified whether n-3 PUFA supplementation may resolve inflammation following environmental stimuli, for example, exercise interventions, through the modification of DNA methylation.

2.4 Impact of n-3 PUFA supplementation on exercise

To our knowledge, no study has investigated whether the exercise induced methylation response can be modulated by n-3 PUFA supplementation; however, there is a wealth of data which has investigated the impact of n-3 PUFA supplementation on measures of exercise performance and the molecular processes which are regulated by both aerobic and resistance exercise.

The supplementation of the diet with n-3 PUFAs has been demonstrated with an anti-inflammatory phenotype and reduce the levels of inflammatory cytokines (Calder 2015; Tartibian et al. 2011; Vedin et al. 2008, 2012), partially as a result of altered DNA methylation (Aslibekyan et al. 2014; Ma et al. 2016). Studies investigating the impact of n-3 PUFAs on exercise phenotypes typically investigate the impact on exercise-induced inflammation; however, the results are equivocal. Some studies have detected reductions in inflammation post-exercise with FA supplementation (Marques et al. 2015; Mickleborough et al. 2015), whereas, others have reported no change in inflammation (Martorell et al. 2014; Nieman et al. 2009). The literature has also shown that n-3 PUFA supplementation reduces oxygen consumption (Kawabata et al. 2014; Peoples et al. 2008), heart rate (Peoples et al. 2008) and perceived exertion (Kawabata et al. 2014) during aerobic exercise suggesting n-3 PUFA supplementation may impact physiological variables.

A common finding of both acute exercise and exercise training studies is the hypomethylation *PPARGC1A* and subsequently, increase the mRNA expression following exercise as a mechanism to induce mitochondrial biogenesis (Barrès et al. 2012; Nitert et al. 2012). Evidence also exists for the ability of n-3 PUFA supplementation to increase the expression of *PPARGC1A* (Hancock et al. 2008; Rundblad et al. 2018) and increase mitochondrial biogenesis (Laiglesia et al. 2016; Turner et al. 2007). These data suggest that n-3 PUFAs may increase exercise performance by upregulating mitochondrial biogenesis; however, no studies to date have investigated whether this occurs via hypomethylation of the gene promoter.

Supplementation of n-3 PUFAs has also been demonstrated to sensitise skeletal muscle to the anabolic stimuli of resistance exercise and protein ingestion (Philpott,

Witard, and Galloway 2018; Tachtsis, Camera, and Lacham-Kaplan 2018). An anabolic role for n-3 PUFAs is suggested by increases in force production following n-3 PUFA supplementation compared to a control supplementation of corn oil (Smith et al. 2015) and a training only group (Rodacki et al. 2012). Another important role for n-3 PUFAs and skeletal muscle growth is the ability to reduce systemic levels of inflammation which have been demonstrated to reduce the regenerative capacity of muscle. The administration of TNF-α on cells has been demonstrated to hypermethylate *MyoD* and reduce markers of differentiation (Sharples et al. 2016). A potential epigenetic consequence for the anabolic role of n-3 PUFAs is highlighted by the administration of EPA to skeletal muscle cells can increase the expression of *PPARGC1A* (Tachtsis et al. 2018) and dampen the effects of TNF-α (Saini et al. 2017), resulting in improved expression of *MyoD* and *Myogenin* indicating increased skeletal muscle differentiation (Saini et al. 2017). The hypermethylation of *MyoD* following the administration of TNF-α, suggests a potential mechanism of n-3 PUFAs to reverse epigenetic changes associated with inflammation in skeletal muscle.

As has been demonstrated above both exercise and n-3 PUFA supplementation have epigenetic consequences through modulated DNA methylation. The pathways regulated by these interventions are similar indicating there may be some overlap in the health benefits of exercise and n-3 PUFA supplementation. To date, there is a lack of studies investigating the modulation of DNA methylation induced by exercise and the supplementation of n-3 PUFAs to determine whether the interventions have any combined impact increasing the epigenetic response.

2.5 Thesis aims

The primary aims of this thesis were to:

1. Identify the impact of different acute exercise stimuli (aerobic and resistance) on global and gene-specific methylation.

2. Ascertain whether the impact of acute exercise can further be modulated by the supplementation of dietary fatty acids.

3. Determine whether the expression of DNMT enzymes are associated with the modulation of DNA methylation in an attempt to identify a potential underlying mechanism.

4. Determine whether modulated DNA methylation as a result of the previously mentioned interventions is associated with physiological markers.

Chapter 3 - General methods

3.0 General methods

This chapter describes the experimental procedures which have been utilised throughout the thesis. The experimental protocol for each study has been approved by the Loughborough University Ethics Human Participants sub-committee and performed in accordance with the Declaration of Helsinki (1975).

3.1 Participants

Before participation, participants were provided with a participant information sheet explaining the purpose, protocols and requirements of the study. Following a verbal explanation and the opportunity to ask any questions about the study, informed consent (Appendix B) was obtained from all participants. Participants also completed a health screen (Appendix C) and a physical activity questionnaire (Appendix D) to ensure suitability to complete the study. All participants were healthy, non-smoking males with no history of metabolic or cardiovascular disease. In the six months prior to the study, participants had no history of n-3 PUFA, anti-oxidant or anti-inflammatory supplementation and habitually consumed less than two portions of oily fish per week.

3.2 Pre-trial measures

3.2.1 Anthropometry

Height was measured, using a stadiometer, to the nearest 0.1 cm with shoes and socks removed. Body mass was assessed, using digital scales, to the nearest 0.1 kg following the removal of shoes and any excess clothing. Body mass index was calculated using the following equation: body mass (kg) / height $(m)^2$.

3.2.2 Pre-trial standardisation

Participants recorded dietary intake (food and drink; Appendix E) during the 24-hours prior to the first trial for each experimental study. For the resistance exercise study (Chapter 7) participants were also required to record dietary intake throughout the trial until the 48-hour post-exercise time point. These diet and physical activity patterns were then repeated for all subsequent trials. Alcohol consumption and exercise was not permitted for the 24-hours preceding and during the pre-trial period. On the morning of experimental trials, participants arrived at the laboratory after a minimum

of a 10 hr fast (only water permitted). The same time of day was used for each trial to minimise diurnal variation. Participants were requested to maintain their habitual diet and physical activity patterns between trials.

3.3 Collection of biological samples

After selection of a vein in the antecubital fossa region, blood samples were obtained by either venepuncture (Chapter 5) or intravenous cannula (Chapters 6 & 7). The cannula was flushed, between sample collections (every 30 min) and after each sample, with saline (0.9% sodium chloride) to prevent blockages forming. Blood was collected into either K_2EDTA coated vacutainers (BD, USA), silica coated vacutainers (BD, USA) or Tempus Blood RNA tubes (Applied Biosystems, USA).

Blood collected into K_2EDTA vacutainers was aliquoted for DNA extraction (Chapters 5-7), RNA extraction (Chapter 7), peripheral blood mononuclear cell (PBMC) extraction (Chapter 6) and whole blood cell counts using the COULTER® Ac·T™ 5diff (Beckman Coulter, USA; Chapters 5 & 6) or the Yumizen H500 (Horiba Medical, Japan; Chapter 7). In chapters 5 & 6, blood was collected into Tempus Blood RNA tubes (Applied Biosystems, USA) for RNA extraction. Blood collected into silica coated vacutainers was allowed to clot for 20 min at room temperature before being centrifuged at 2800 rpm for 15 min at 4 ^{o}C. The layer of serum was collected and stored at -80˚C prior to analysis of inflammatory cytokines (Chapters 6 & 7), protein carbonyls (Chapter 6) and markers of muscle damage (Chapter 7).

3.4 Processing and analysis of biological samples

3.4.1 DNA extraction and bisulfite conversion

For chapters 5 & 6, genomic DNA (gDNA) was extracted from 2 mL of whole blood using the QIAamp DNA Blood Midi kit (Qiagen, Germany) according to the manufacturer's instructions (Appendix Fi). Extracted gDNA was aliquoted and stored at -20 ^{o}C until required for further analysis. Bisulfite conversion of gDNA samples (maximum 2µg) was performed using the EpiTect Fast Bisulfite Conversion Kit (Qiagen, Germany) according to the manufacturer's instructions (Appendix Gi).

Bisulfite-converted DNA (bcDNA) was stored at -20 °C until required for further analysis.

For samples collected in the resistance exercise study (Chapter 7), DNA was extracted from 200 µL of whole blood using the ReliaPrep gDNA Blood Miniprep System according to the manufacturer's instructions (Promega, USA; Appendix Fii) for assessment of global DNA methylation. DNA extraction and bisulfite conversion of both whole blood and skeletal muscle was performed using the EpiTect Fast LyseAll Bisulfite Kit (Qiagen. Germany) according to the manufacturer's instructions (Appendix Gii) and stored at 20 °C until required for further analysis. DNA yield (ng/µL) and purity (absorbance ratio A_{260}/A_{280}) were determined using a Nanodrop 2000 (ThermoScientific, USA). The mean (± standard deviation) DNA concentration and purity are described in the method for individual experimental chapters.

3.4.2 Luminometric Methylation Assay (LUMA)

The Luminometric Methylation Assay (LUMA) was used as a marker of global DNA methylation as previously described (Karimi et al. 2006), with minor adjustments. Two mastermixes were set up per sample, one containing FastDigest *Hpa*II and the other FastDigest *Msp*I (Thermo Scientific, USA). Table 3.1 lists the volume of each reagent used for the mastermixes. The master mix was then added to 200 ng of gDNA and incubated for 20 min at 37˚C.

Table 3.1 – *Volumes of reagents used to create the mastermixes for the Luminometric Methylation Assay (LUMA). Two mastermixes were created per sample, one containing HpaII and the other MspI.*

Reagent	Volume per sample (µL)
*Hpa*II / *Msp*I	0.25 µL
*Eco*RI	0.25 µL
Reaction Buffer	1 µL
Water	3.5 µL
Total	5 µL

Following incubation, 13 µL of each reaction were mixed with annealing buffer and added to a separate well of a PyroMark Q24 plate and analysed using a PyroMark Q24 MDx system (Qiagen, Germany) with the following dispensation order: ACTCGA. The volumes of reagents added to the Q24 cartridge are indicated below (Table 3.2). Peak heights were exported, and methylation percentage was calculated using the following formula:

Methylation = (1 - (HpaII peak 2 / HpaII peak 1) / (MspI peak 2 / MspI peak 1)) x 100.

Table 3.2 - *Volumes of reagents added to the chambers of the PyroMark Q24 cartridge for the Luminometric Methylation Assay (LUMA)*

Chamber	Volume of reagent
A	30 µL dATPαS and 30 µL nuclease-free water
C	30 µL dCTP and 30 µL dGTP
G	60 µL nuclease-free water
T	30 µL dTTP and 30 µL nuclease-free water
E	80 µL Enzyme
S	80 µL Substrate

3.4.3 Gene-specific Pyrosequencing

Polymerase chain reaction (PCR) of bisulfite-converted DNA (bcDNA) samples was performed using the PyroMark PCR Kit (Qiagen, Germany) according to the manufacturer's instructions. The mastermix recipe (Table 3.3) and PCR cycling conditions are indicated below (Table 3.4). PCR products were analysed by gel electrophoresis on a 2 % agarose gel with SYBRsafe (Invitrogen, USA) and visualised by ultraviolet trans-illuminator (BioRad, USA) to confirm the generation of a single high-quality PCR product and no contamination in negative control samples. The absence of PCR amplification of a non-bisulfite converted DNA sample confirmed the specificity of each assay for bisulfite-converted DNA.

DNA methylation was assessed using a PyroMark Q48 Autoprep system (Qiagen, Germany) using PyroMark Q48 Advanced CpG Reagents (Qiagen, Germany) according to the manufacturer's instructions. Custom PyroMark CpG assays were designed to assess DNA methylation at specific CpG sites. Genomic location, primer sequences and the sequence to analyse for the assays are presented in experimental chapters, and details on assay validation are provided in Chapter 4 (Method Development). The nucleotide dispensation order was generated by entering the sequence to analyse into the PyroMark Q48 Autoprep software version 2.4.2 (Qiagen, Germany). A non-CpG cytosine was included in the nucleotide dispensation order next to a bisulfite-converted thymine to detect incomplete bisulfite conversion. The methylation at each CpG site was determined using the PyroMark Q48 Autoprep software set in CpG mode. The mean methylation of all CpG sites within the target region was determined using the methylation at the individual CpG sites.

Table 3.3 - *Volume of reagents (per sample) used to create a PCR mastermix for pyrosequencing assays.*

Reagent	Volume per sample
PyroMark PCR Mastermix	12.5 µL
CoralLoad concentrate	2.5 µL
PyroMark custom assay PCR primer	2.5 µL
ddH2O	6.5 µL
bcDNA	1 µL
Total	25 µL

Table 3.4 - *PCR cycling conditions utilised for pyrosequencing assays.*

Stage	Time	Temperature	Cycling
Initial activation	15 min	95°C	N/A
Denaturation	30 s	95°C	x45 cycles
Annealing	30 s	56°C	
Extension	30 s	72°C	
Final extension	10 min	72°C	N/A

3.4.4 Correction for blood cell heterogeneity

Within a tissue, the most important factor in determining DNA methylation is the composition of cell types. Changing populations of white blood cells (WBC) in whole blood between time points may confound the assessment of DNA methylation. Whole blood cell counts were used to adjust DNA methylation, to account for different cell populations between time points, using a regression-based approach (Jones et al. 2017). In brief, a linear regression was fitted to the DNA methylation with the populations of WBC (neutrophils, lymphocytes, monocytes, eosinophils and basophils) entered as additive variables. The residuals of the observed DNA methylation value and the predicted methylation from the linear regression were extracted and added to the mean methylation of the dataset. This adjustment decorrelates the methylation percentage from each of the WBC populations to remove the impact of different quantities of WBC populations on DNA methylation to allow comparison of DNA methylation between time points.

3.4.5 RNA extraction and cDNA conversion

In chapters 5 & 6, RNA was isolated from whole blood collected in Tempus Blood RNA tubes using the Tempus Spin RNA Isolation Kit (Applied Biosystems, USA) according to the manufacturer's instructions (Appendix Hi). In Chapter 7, RNA was extracted from skeletal muscle and whole blood using TRI Reagent (Sigma-Aldrich, USA) and TriZol LS (Invitrogen, USA) respectively, according to manufacturer's instructions (Appendix Hii). RNA yield (ng/µL) and purity (absorbance ratio A_{260}/A_{280}) were determined using a Nanodrop 2000 (ThermoScientific, USA). The optimal absorbance ratio is ~2.0, the mean (± standard deviation) ratio are described in the method for individual experimental chapters.

A maximum of 2 µg of RNA was reverse transcribed into complementary DNA (cDNA) using the High-Capacity Reverse Transcription Kit (Applied Biosystems, USA) and diluted to a concentration of 5 ng/µL in deionised water. The mastermix reagents (Table 3.5) and cycling conditions (Table 3.6) used for the cDNA synthesis are displayed below.

Table 3.5 - *Volume of reagents used to create the mastermix for cDNA synthesis.*

Reagent	Volume per sample
RT Buffer	2.0 µL
dNTP Mix (100 mM)	0.8 µL
Random Primers	2.0 µL
MultiScribe Reverse Transcriptase	1.0 µL
RNase Inhibitor	1.0 µL
Nuclease-free water	3.2 µL
Total	10.0 µL

Table 3.6 - *PCR cycling conditions utilised for cDNA synthesis*

Stage	Time	Temperature
1	10 min	25 °C
2	120 min	37 °C
3	5 min	85 °C

3.4.6 mRNA expression

A Viia7 Real-Time PCR system (Applied Biosystems, USA) was used for analysis of mRNA expression. Each reaction contained 5 µL of PrecisionPlus Sybr Green Master Mix (PrimerDesign, UK), 0.5 µL of forward and reverse primer (10 µM) and 4 µL of cDNA (normalised to 5 ng/µL). Primer sequences for mRNA expression assays are presented experimental chapters, and details on assay validation are detailed in Chapter 4 (Method development). All samples were run in duplicate using a Viia7 Real-Time PCR system (Applied Biosystems, USA) using the following cycling conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A melt curve was then run from 60 °C to 95 °C at a rate of 0.05 °C/s. Melt curves were visually inspected for a single peak indicating the generation of a single product.

Relative mRNA expression was performed by quantitative PCR (qPCR) for each gene of interest, normalised to the expression of the reference gene *GAPDH* using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). The following equation was used to determine mRNA expression:

Relative quantification = $2^{-(\Delta Ct\ Sample) - (\Delta Ct\ Control)}$; where ΔC_t = C_t of target gene - C_t of reference gene. The pooled group mean of the pre-exercise sample of the initial trial was used as the control. The mean Ct value (± standard deviation) of GAPDH value across all participants and experimental conditions is described in individual experimental chapters.

3.5 Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics software version 23 (SPSS; IBM, USA). The data distribution was assessed for normality by Shapiro-Wilk's test ($p > 0.05$). Repeated measures Analysis of Variance (RM-ANOVA) or t-tests (where appropriate) were used to determine the impact of exercise and FA supplementation on DNA methylation, mRNA expression and physiological variables. When Mauchly's assumption of sphericity was violated, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Where significant effects were observed, the Bonferroni correction was used to control the familywise error rate. Data sets were determined to be significantly different when $p < 0.05$. Analysis of DNA methylation data was conducted following the correction for cell heterogeneity (section 3.4.4). Analysis of relative mRNA expression was performed on log-transformed fold change data and presented as back-transformed mean with confidence intervals (CI). Unless stated otherwise, data are presented as mean ± 95% CI.

Spearman's Rho correlation analysis was used to assess the relationship between DNA methylation values, mRNA expression values and physiological markers. Moderate (>0.5) and large (> 0.7) correlation coefficients were considered to be of interest; however, only correlations with a p-value < 0.05 were deemed statistically significant.

Chapter 4 Method development - Selection and laboratory standardisation of DNA methylation and mRNA expression methods.

4.1 Introduction

4.1.1 Selection of DNA methylation methods

In general, DNA methylation methods can be divided into three broad categories: global DNA methylation, genome-wide methylation and site-specific methylation. The selection of which method used to analyse methylation is dependent on a range of different factors including the aim of the study, the quantity of DNA, available equipment and the cost involved. This section reviews some of the different methods which can be used to detect DNA methylation to select the methods which will be utilised throughout this thesis.

4.1.1.1 Global DNA methylation methods

Global DNA methylation can be used to gain insight into the impact of an intervention on DNA methylation patterns. Global based methods do not allow the investigation of specific biological pathways; however, altered global DNA methylation has been associated with several disease states including various forms of cancer (Gao et al. 2014; Joyce et al. 2016), rheumatoid arthritis (Liu et al. 2011) and cardiovascular disease (Kim et al. 2010). Aside from disease models, global methylation has also been used to determine the impact of lifestyle interventions including exercise (see section 2.2) and nutrition (see section 2.3).

The gold-standard method for the highly sensitive determination of global methylation is using high-performance liquid chromatography (HPLC; Kuo et al. 1980). Although the gold-standard method, HPLC requires the use of specialist equipment and large quantities of DNA making it an impractical option to be used in the present thesis. Another highly sensitive method to determine global methylation is using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS; Song et al. 2005). Unlike HPLC, LC-MS/MS only requires a small quantity of DNA to determine methylation; however, the requirement of specialist equipment prevented the use of this method. Unlike HPLC and LC-MS/MS, the use of enzyme-linked immunosorbent assay (ELISA) based DNA methylation detection does not require any specialist equipment to determine global DNA methylation (Kurdyukov and Bullock 2016). Despite the lack of specialist equipment required, So et al. (2014) report ELISA based detection may only be suitable when identifying large variations in global DNA

methylation because of high assay variability; therefore, the use of ELISA for the may lack the sensitivity to determine small changes in methylation which are expected in this thesis.

After ruling out the use of the methods above, the remaining options to determine global DNA methylation are via the methylation of the LINE-1 sequences or LUMA as surrogates of global DNA methylation. LINE-1 sequences comprise ~17% of the human genome and hypomethylation of LINE-1 sequences have previously been associated with cancer (Gao et al. 2014). LINE-1 DNA methylation is strongly correlated with the gold-standard global methylation technique HPLC suggesting it is a good estimate for global DNA methylation (Lisanti et al. 2013). Initially pyrosequencing of LINE-1 elements was selected as the method to determine global methylation; however, the commercially available LINE-1 pyrosequencing failed the validation procedure because of low peak heights (Appendix I).

The lack of a functioning LINE-1 assay meant LUMA was selected as the method to determine global DNA methylation in this thesis. LUMA quantifies the methylation of internal CpG sites within CCGG sequences throughout the genome (Karimi et al. 2006). In total, CCGG sequences account for ~8% of the total CpG sites throughout the human genome (Fazzari and Greally 2004). These CpG sites occur in both repetitive element and protein coding regions (Fazzari and Greally 2004), whereas, LINE-1 sequences are only situated in nongenic regions. To perform LUMA, DNA is digested in two reactions; one incubated with the methylation-sensitive enzyme HpaII and the other with MspI which digests regardless of methylation status. EcoRI is added to each reaction to control for the input quantity of DNA. The digested reactions are pyrosequenced, and the overhangs filled by nucleotides producing light. DNA methylation is then determined by the HpaII / MspI ratio (Karimi et al. 2006).

4.1.1.2 Gene-specific DNA methylation methods

The gene-specific determination of DNA methylation can be divided into two general categories, genome-wide methods and candidate-gene focussed approaches. Genome-wide based detection methods are useful for the determination of differentially methylated regions of the genome at single-base resolution; however,

these methods are highly expensive and require the use of specialist equipment which was not available for this thesis. Candidate-gene based approaches allow the determination of methylation at specific CpG sites using PCR to amplify the region of interest; however, DNA needs to be processed (bisulfite converted) before PCR amplification because methylation is not maintained during PCR.

4.1.1.2.1 Bisulfite conversion of DNA for methylation analysis

The bisulfite conversion of DNA involves the chemical modification of cytosine into uracil via a 3-step process of sulfonation, deamination and desulfonation (Figure 4.1). Bisulfite conversion does not alter methylated DNA, therefore, creating sequence differences between methylation and unmethylated DNA (Table 4.1; Hernández et al. 2013; Delaney, Garg, and Yung 2015). During PCR uracil pairs with adenine in the first cycle, then in the remaining cycles adenine will bind with thymine; therefore, the uracil bases are amplified as thymine, whereas, methylated cytosines remain as cytosine (Table 4.1). The proportion of cytosine/thymine can then be used to determine DNA methylation percentage (Hernández et al. 2013).

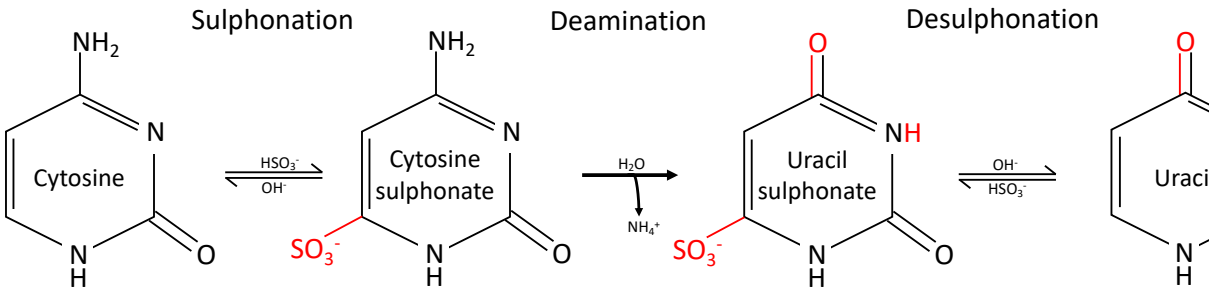

Figure 4.1 - *Stages involved in the bisulfite conversion process.*

Several PCR-based methods have been developed to determine DNA methylation including methylation-specific PCR (Herman et al. 1996), COBRA (Xiong and Laird 1997), MethyLight (Eads et al. 2000), and methylation-sensitive high-resolution melt (Wojdacz and Dobrovic 2007). The drawback of many of these PCR based techniques is they can only determine the overall methylation of a PCR product (i.e. they do not allow the investigation of methylation of individual CpG site). Sequencing of PCR products can be used to determine the methylation status of individual CpG sites within the PCR product (Kurdyukov and Bullock 2016). The limitation of sequencing-based methods is the access to a DNA sequencer; however, within our laboratory, we have

access to a pyrosequencer which can be used to determine the methylation percentage of individual CpG sites within a PCR product.

Table 4.1 - *Bisulfite conversion induce sequence differences between methylated and unmethylated DNA.*

	Unmethylated DNA	Methylated DNA
Original Sequence	T-G-A-**C**-**C**-G-A-**C**-G-**C**	T-G-A-**C**-**^{m}C**-G-A-**^{m}C**-G-**C**
Bisulfite converted sequence	T-G-A-**U**-**U**-G-A-**U**-G-**U**	T-G-A-**U**-**^{m}C**-G-A-**^{m}C**-G-**U**
PCR product	T-G-A-**T**- **T**-G-A-**T**-G-**T**	T-G-A-**T** - **C** -G-A- **C** -G-**T**

4.1.1.2.2 Pyrosequencing based DNA methylation detection

Pyrosequencing is one of the most commonly used methods to determine DNA methylation accurately at specific CpG sites. Originally pyrosequencing was developed to identify SNPs; however, after bisulfite conversion, pyrosequencing can be used to determine the ratio of the bisulfite-converted C/T SNP (Delaney et al. 2015; Tost, Dunker, and Gut 2003)

Following bisulfite conversion, DNA undergoes PCR with one of the primers labelled with biotin. The biotin labelled PCR product then binds to streptavidin-coated beads and DNA is denatured creating single-stranded DNA. The unlabelled DNA strand is washed away, leaving the labelled strand free to be sequenced. A sequencing primer is then introduced, and nucleotides are dispensed in a specific order to be incorporated by DNA polymerase (Harrington et al. 2013). The incorporation of nucleotides releases pyrophosphatase which can be converted into light by an enzyme cascade (Figure 4.2). The amount of light produced is proportional to the amount of pyrophosphatase generated and therefore the number of nucleotides incorporated (Delaney et al. 2015; Harrington et al. 2013; Tost and Gut 2007). DNA methylation can be quantified by the amount of light produced by the incorporation of cytosine (methylated DNA) compared to thymine (unmethylated bisulfite-converted cytosine).

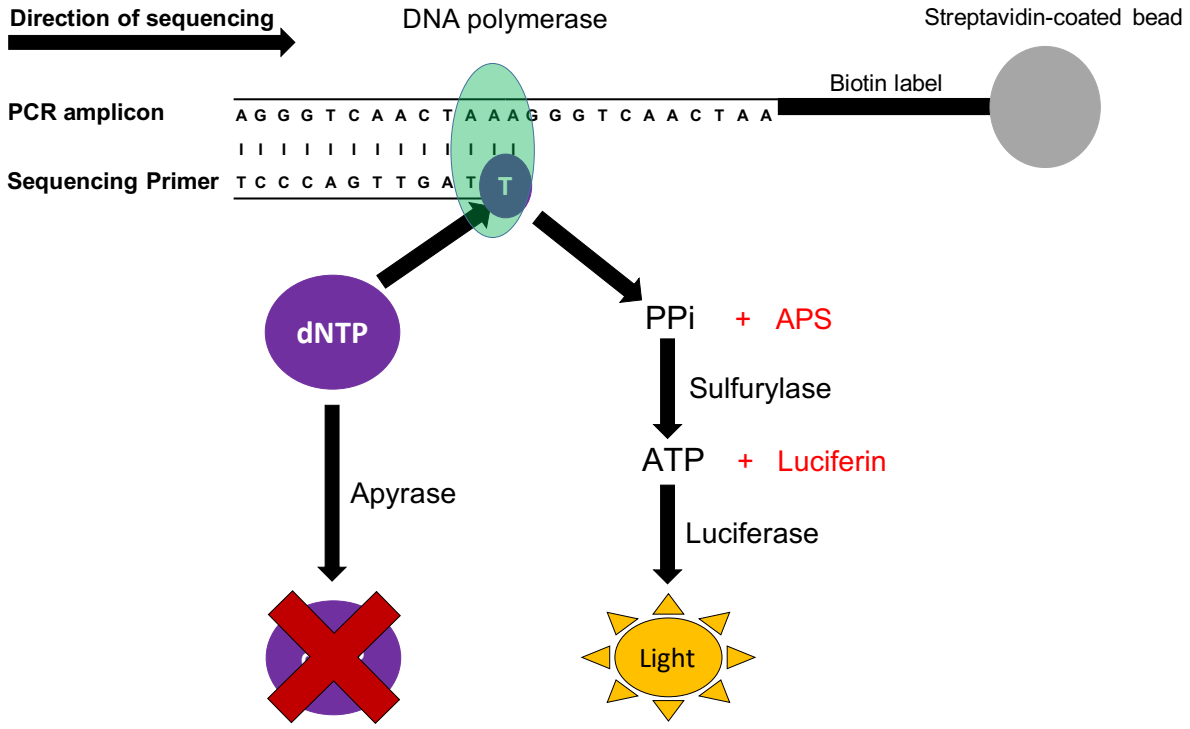

Figure 4.2 – *Pyrosequencing enzyme cascade. Biotin labelled primer binds to streptavidin-coated beads, DNA is then denatured, and the sequencing primer binds to the labelled DNA strand. Nucleotides are then dispensed and incorporated into the sequenced DNA strand releasing PPi. PPi is then converted into ATP in a reaction using the substrate APS catalysed by the enzyme Sulfurylase. ATP in the presence of the substrate luciferin is then converted into oxyluciferin and light by the enzyme luciferase. The amount of light produced is proportional to the number of nucleotides incorporated. Any unincorporated nucleotide is degraded by apyrase before the dispensation of the next nucleotide. dNTP, nucleotides; PPi, pyrophosphate; APS, adenosine phosphosulfate; ATP, adenosine triphosphate.*

4.1.2 Characterisation of DNA methylation and mRNA expression assays

Throughout this thesis, the impact of exercise and n-3 PUFA supplementation on DNA methylation and mRNA expression is examined. The global DNA methylation analysis in this thesis utilises LUMA which is a well-established method; however, gene-specific DNA methylation is investigated using custom pyrosequencing assays. It is essential to validate these custom-assays to ensure they accurately determine the DNA methylation of the CpG they have been designed to evaluate.

When using custom-designed pyrosequencing assays to detect DNA methylation, the specificity and efficiency of assays must be determined to ensure accurate and precise quantification (Delaney et al. 2015). The first stage in assay validation is to confirm the

amplification of a single PCR amplicon free of primer dimer and artefacts which can inhibit pyrosequencing (Noehammer et al. 2014). The amplification of non-bisulfite converted DNA during PCR causes inaccurate quantification of DNA methylation because of an overrepresentation of cytosines (Izzi, Binder, and Michels 2014); therefore, it is essential to confirm the PCR amplification of bcDNA and absence of gDNA amplification.

After the quality of the PCR product has been confirmed, it is essential to validate the efficiency of the PCR reaction is not altered by sequence differences created by the bisulfite conversion process (Hernández et al. 2013). The differing cytosine content may create PCR bias towards the amplification of either methylated or unmethylated template DNA potentially leading to the overestimation or masking of DNA methylation (Warnecke et al. 1997). The presence of PCR bias can be determined by performing pyrosequencing with standards of a known DNA methylation content (Delaney et al. 2015; Warnecke et al. 1997).

The functional consequence of altered DNA methylation is evaluated via the measurement of mRNA expression (Jones 2012). The reverse transcription of RNA to cDNA followed by qPCR (RT-qPCR) is one of the most commonly used and sensitive methods for the detection of mRNA expression. Reactions are quantified by the point in which the fluorescence is detected above the baseline, termed cycle threshold (C_t). The amount of fluorescence detected is proportional to the quantity of PCR product. A lower C_t value indicates the generation of more template; therefore, relative quantification of one sample to another can be performed by comparing C_t values. To account for differences in the input cDNA between samples the quantity of target mRNA of each target is normalised to the quantity of a stably expressed reference (Valasek and Repa 2005).

The amplification efficiency of an assay is critical for accurate quantification (Wong and Medrano 2005). The optimal efficiency of 100% indicates the doubling of cDNA with each cycle during the linear phase of qPCR (Svec et al. 2015). When using the comparative Ct ($2^{-(\Delta\Delta Ct)}$) method for relative quantification, small deviations from optimal efficiency are tolerable as long as the efficiencies between the target and

reference are approximately equal (Livak and Schmittgen 2001). Amplification efficiency and assay linearity can be estimated by the creation of a standard curve using the serial dilution of a cDNA sample (Bustin and Huggett 2017; Kuang et al. 2018).

The specificity of an mRNA expression assay for the desired template is also critical for accurate quantification. When using DNA binding dyes, including SYBR Green-based detection, the generation of any double-stranded DNA produces fluorescence (Wong and Medrano 2005). Melt curves can determine assay specificity by detecting the loss in fluorescence caused by the disassociation of DNA strands with increasing temperature (Kuang et al. 2018). The generation of a single peak on a melt curve indicates the amplification of a distinct PCR product (Ririe, Rasmussen, and Wittwer 1997).

Non-validated DNA methylation and mRNA expression assays can cause inaccurate results; therefore, it is essential to confirm assay specificity and linearity before using the assays to quantify DNA methylation or mRNA expression. The performance of the assays used to determine DNA methylation, and mRNA expression are evaluated in this chapter to ensure accurate quantification.

4.2 Methods

4.2.1 Pyromark CpG methylation assays

4.2.1.1 Design of CpG Methylation assays

Promoter and first exon sequences for genes of interest were investigated for CpG sites using the UCSC genome browser and existing literature. Once target regions were identified, the FASTA DNA sequence for each target gene was downloaded from Ensembl (National Centre for Biotechnology Information (NCBI) build 38) and imported into the PyroMark Assay Design software 2.0 (Qiagen, Germany). The target region of each gene was selected and the Pyromark Assay Design software designed assays (PCR and sequencing primer sets) and scored the quality of the assays out of 100. The assay with the highest score was selected, and the primer sequences were then checked for sequence similarity against other gDNA sequences using Primer-BLAST (Ye et al. 2012) and bcDNA sequences using BiSearch (Tusnady et al., 2005). The genomic location, primer sequences (PCR and sequencing) and sequence to analyse for the pyrosequencing assays used throughout this thesis are presented in Table 4.2. Appendix J contains full details of pyrosequencing assays including sequence, assay design report, nucleotide dispensation order and example pyrograms.

4.2.1.2 Validation of CpG Methylation assays

The EpiTect PCR control DNA set (Qiagen, Germany) was used to create standards of bcDNA of known methylation percentages (0%, 12.5%, 25%, 50%, 75%, 87.5%, 100%). Standards for each pyrosequencing assay underwent PCR, gel electrophoresis and pyrosequencing as previously described (section 3.4.3). Gel electrophoresis images were visually inspected to confirm the specificity of the PCR (Figure 4.4). Pyrograms generated during pyrosequencing were inspected and data extracted to determine the methylation percentage of the standards (Figure 4.3; Appendix J). Standard curves were generated between the observed and expected DNA methylation to check the assays for PCR bias.

Table 4.2 – *Details of pyrosequencing assays used to determine DNA methylation throughout this thesis. Genomic location identified using Genome Reference Consortium Human Build 38. CpG sites are indicated in the sequence to analyse by* ***Y****. TSS, transcription start site; bp, base pair.*

Assay ID [Genomic location]	Primer	Sequence	No. of CpG sites (distance from TSS; bp)
IL6 [chr7:22726051 - 22726198]	Forward:	5'-GGGAAGAGGGTTTTTGAATTAG-3'	6 (-1099, -1096, -1094, -1069, -1061 & -1057)
	Reverse:	5'-biotin-CTCCCTCTCCCTATAAATCTTAATTTAA-3'	
	Sequencing:	5'-TTGAATTAGTTTGATTTAATAAGAA-3'	
	Sequence to analyse:	ATTTTTGGGTGTYGAYGYGGAAGTAGATTTAGAGTTTAGAGTYG TGTTTGYGTTYGTAGTTTTTTTTTAGTTTTTTTTGATTT	
TNF [chr6:31575730 - 31575816]	Forward:	5'-GGAAAGGATATTATGAGTATTGAAAGTATG-3'	4 (+197, +202, +214 & +222)
	Reverse:	5'-biotin-CTAAAACCCCCCTATCTTCTTAAA-3'	
	Sequencing:	5'-ATTATGAGTATTGAAAGTATGAT-3'	
	Sequence to analyse:	TYGGGAYGTGGAGTTGGTYGAGGAGGYGTTTTTTAAGAAGATA GGGGGGTTT	
PPARGC1A [chr4:23890308 - 23890372]	Forward:	5'-TGTAGGGGATTTTGGTTATTATATGGT-3'	1 (-260)
	Reverse:	5'-biotin-ACCAACTTTAAATACCACAAACTCTA-3'	
	Sequencing:	5'-GGTTATTATATGGTTAGGGT-3'	
	Sequence to analyse:	TTYGTTTAGAGTTTGTGGTATTTAAAGTT	
PPARGC1A ALT [chr4: 23904183 - 23904315]	Forward:	5'-AAGGGAATTATTTGTTTTAATTGTTGATG-3'	3 (-182, -131 & -127)
	Reverse:	5'-biotin-AACACAAATCTAAAACCCAATCT-3'	
	Sequencing:	5'-GTTGATGTTAGAGAGTTTT-3'	
	Sequence to analyse:	TTYGAGATATAGGGTTGTTGGAAAGTATATGATATTGTATATA TTTGTTTTTAYGTTYGTATTTGGTTAA GATTGGGTTT TAGAT	

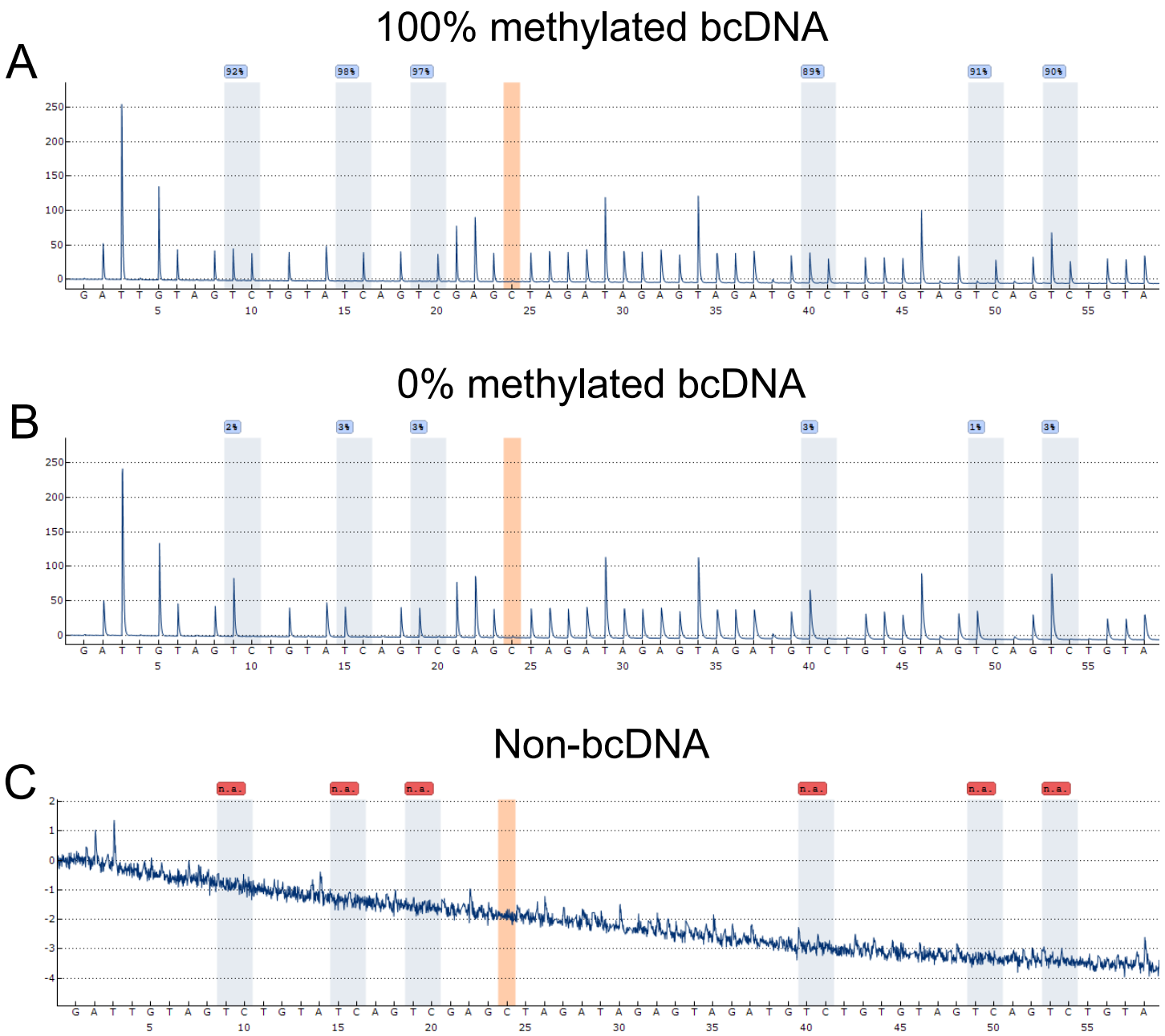

Figure 4.3 *- Example pyrograms for the IL6 pyrosequencing assay showing (A) 100% methylated standard, (B) 0% methylated standard and (C) Non-bisulfite converted DNA. CpG sites are indicated by the greyed-out bar. The percentages in the boxes above indicate the determined methylation percentage. The colour of the box indicates the quality control result (Blue = pass; Red = fail). The orange bar indicates the position of the bisulfite conversion quality control to check for sequencing of non-bisulfite converted unmethylated cytosines.* Pyrograms for the other assays displayed in Appendix J.

4.2.2 mRNA expression assays

4.2.2.1 Design of mRNA expression assays

Previously published primer sets were used for the determination of mRNA expression (Table 4.3). Assays with primers situated at exon junctions or spanning introns were selected, except for the total *PPARGC1A* primer pair, to prevent the amplification of unwanted gDNA in samples. Both primers for total *PPARGC1A* are situated in the same exon to ensure all transcripts were assessed. Primer sequences were input to Primer-BLAST (Ye et al. 2012) to check the specificity of the assay for a single genomic location. Appendix K contains the full sequence of each assay indicating the genomic location.

4.2.2.2 Validation of mRNA expression assays

The efficiency of each mRNA expression assay was determined using standard curves generated from a 2-fold serial dilution of a cDNA sample using the conditions outlined in section 3.4.6.

The efficiency (E) was calculated using the formula:
$E = ((10^{(-1/slope)}) - 1) \times 100$, where the slope is the gradient of the linear regression fitted to the standard curve. Melt curves for each assay were generated according to the conditions outlined in section 3.4.6 to check the specificity of the qPCR for the amplification of a single amplicon.

Table 4.3 - *Primer sequences for the mRNA expression assays used in this thesis*

Assay ID	Accession No.	Primer	Sequence	Amplicon length	Reference
GAPDH	NM_001289745.2	Forward: Reverse:	5'- GCCTCAAGATCATCAGCAATGCCT-3' 5'- TGTGGTCATGAGTCCTTCCACGAT-3'	104	(Korah et al. 2012)
IL6	NM_000600.4	Forward: Reverse:	5'-GCAGAAAAAGGCAAAGAATC-3' 5'-CTACATTTGCCGAAGAGC-3'	178	(Campanelli et al. 2016)
TNF	NM_000594.3	Forward: Reverse:	5'-AGGCAGTCAGATCATCTTC-3' 5'- TTATCTCTCAGCTCCACG-3'	142	(Campanelli et al. 2016)
Total *PPARGC1A*	NM_001330751.1	Forward: Reverse:	5'-CAGCCTCTTTGCCCAGATCTT-3' 5'-TCACTGCACCACTTGAGTCCAC-3'	101	(Popov et al. 2015)
PPARGC1A Exon 1a	NM_013261.4	Forward: Reverse:	5'-ATGGAGTGACATCGAGTGTGCT-3' 5'-GAGTCCACCCAGAAAGCTGT-3'	127	(Silvennoinen et al. 2015)
PPARGC1A Exon 1b	XM_011513766.1	Forward: Reverse:	5'-CTATGGATTCAATTTTGAAATGTGC-3' 5'-CTGATTGGTCACTGCACCAC-3'	153	(Silvennoinen et al. 2015)
DNMT1	NM_001130823.2	Forward: Reverse:	5'-TACCTGGACGACCCTGACCTC-3' 5'-CGTTGGCATCAAAGATGGACA-3'	103	(Wu et al. 2007)
DNMT3a	NM_175629.2	Forward: Reverse:	5'-TATTGATGAGCGCACAAGAGAGC-3' 5'-GGGTGTTCCAGGGTAACATTGAG-3'	111	(Wu et al. 2007)
DNMT3b	NM_006892.3	Forward: Reverse:	5'-GGCAAGTTCTCCGAGGTCTCTG-3' 5'-TGGTACATGGCTTTTCGATAGGA-3'	113	(Wu et al. 2007)

4.3 Results

4.3.1 PyroMark CpG assays

Primer sequences were checked for specificity using Primer-BLAST and BiSearch, and the generation of a single high-quality template was confirmed using gel electrophoresis (Figure 4.4). For each bcDNA standard, a single band was detected indicating PCR product free of artefacts and primer dimer formation which may interfere with pyrosequencing. The absence of amplification of the gDNA sample displayed by the lack of a band in gel electrophoresis (Figure 4.4) and a lack of sequencing on the pyrogram (Figure 4.3C) indicates the specificity of the reaction for bcDNA. A no template control was also electrophoresed to check for contamination with bcDNA, which would impact the determination of methylation percentage.

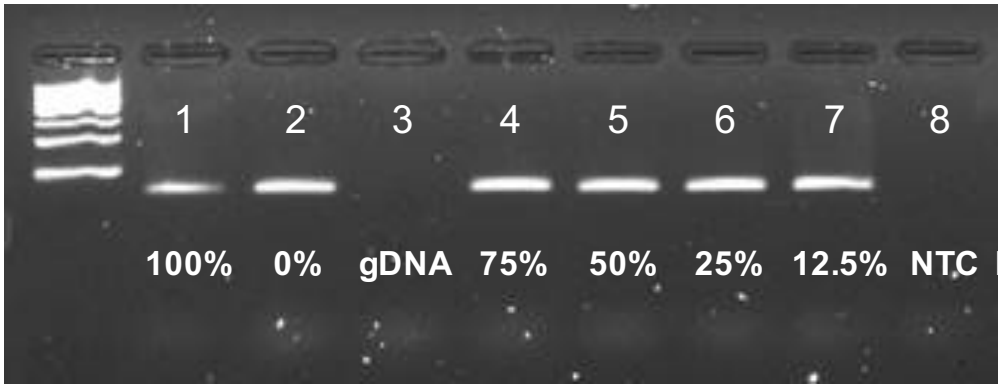

Figure 4.4 - *Example gel electrophoresis image of PyroMark assay PCR product. Left hand lane = 100bp ladder; lanes 1-8 = standards (indicated below lane ID). PCR generated a single product for each of the bcDNA standards; however, no amplification occurred in the gDNA or NTC. gDNA = genomic DNA; NTC = no template control; bcDNA = bisulfite-converted DNA.*

Standard curves, for each pyrosequencing assay, were generated between expected methylation of the bcDNA standards and the mean methylation observed across CpG sites (Figure 4.5; see Appendix L for standard curves of individual CpG sites). A proportional increase in DNA methylation was detected with each standard, and a linear regression was fitted with a high coefficient of determination ($R^2 > 0.99$) for each assay (Figure 4.5). The linearity of these assays across different methylation percentages indicates the absence of PCR bias.

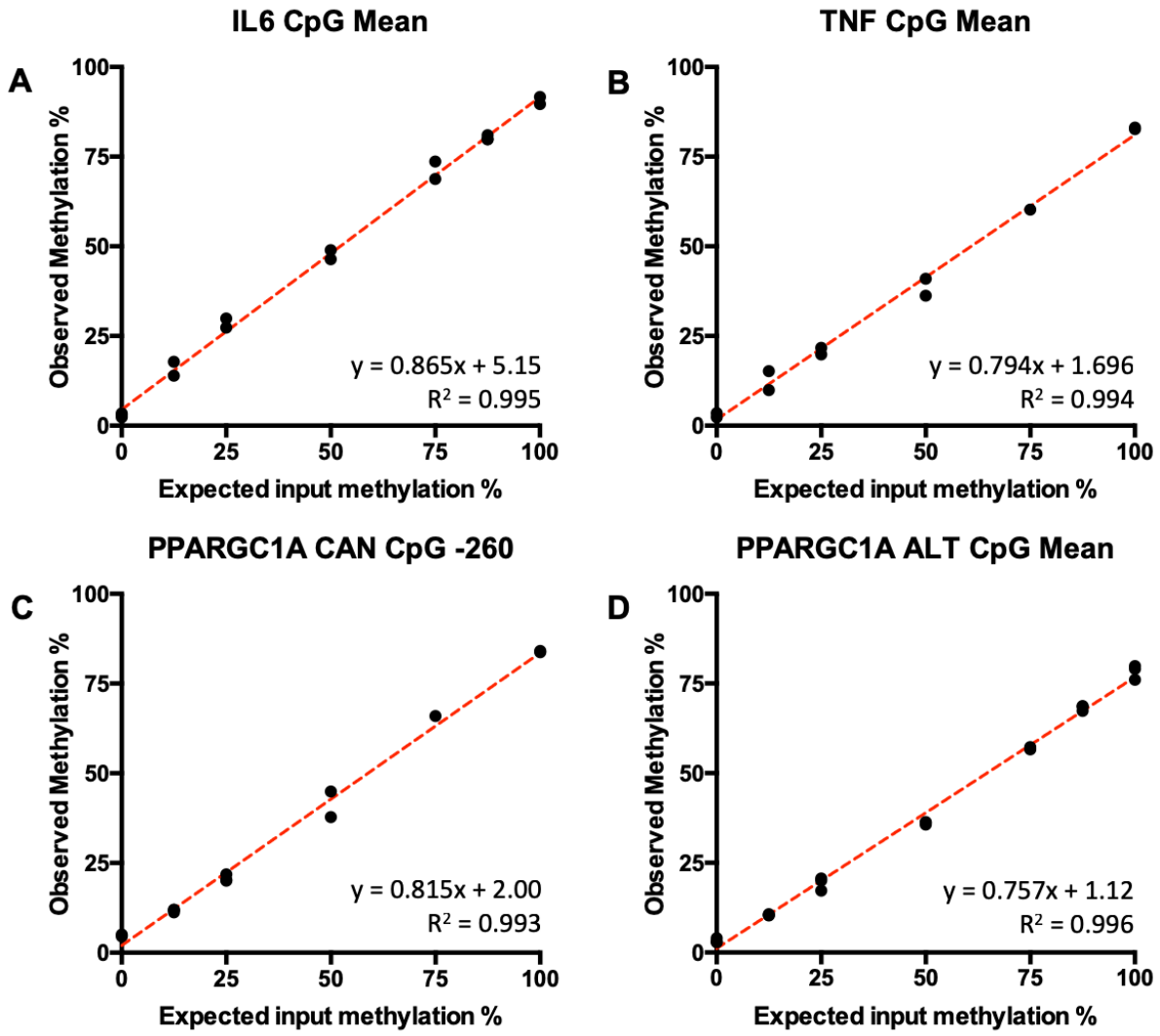

Figure 4.5 - *Standard curves generated between the expected methylation of bcDNA standards and the mean observed methylation. Points indicate the mean methylation of the CpG sites of the (A) IL6, (B) TNF , (C) PPARGC1A CAN and (D) PPARGC1A ALT PyroMark assays. Red dashed line indicates the linear regression curve. The equation of the line and coefficient of determination (R^2) are located in the bottom right corner of each graph.*

4.3.2 mRNA expression assays

Primer-BLAST indicated that only a single amplicon would be generated for each of the primer sets. The specificity of the assays for a single amplicon was confirmed by the generation of a single peak on melt curves (Figure 4.6, individual melt curves for each assay are located in Appendix M).

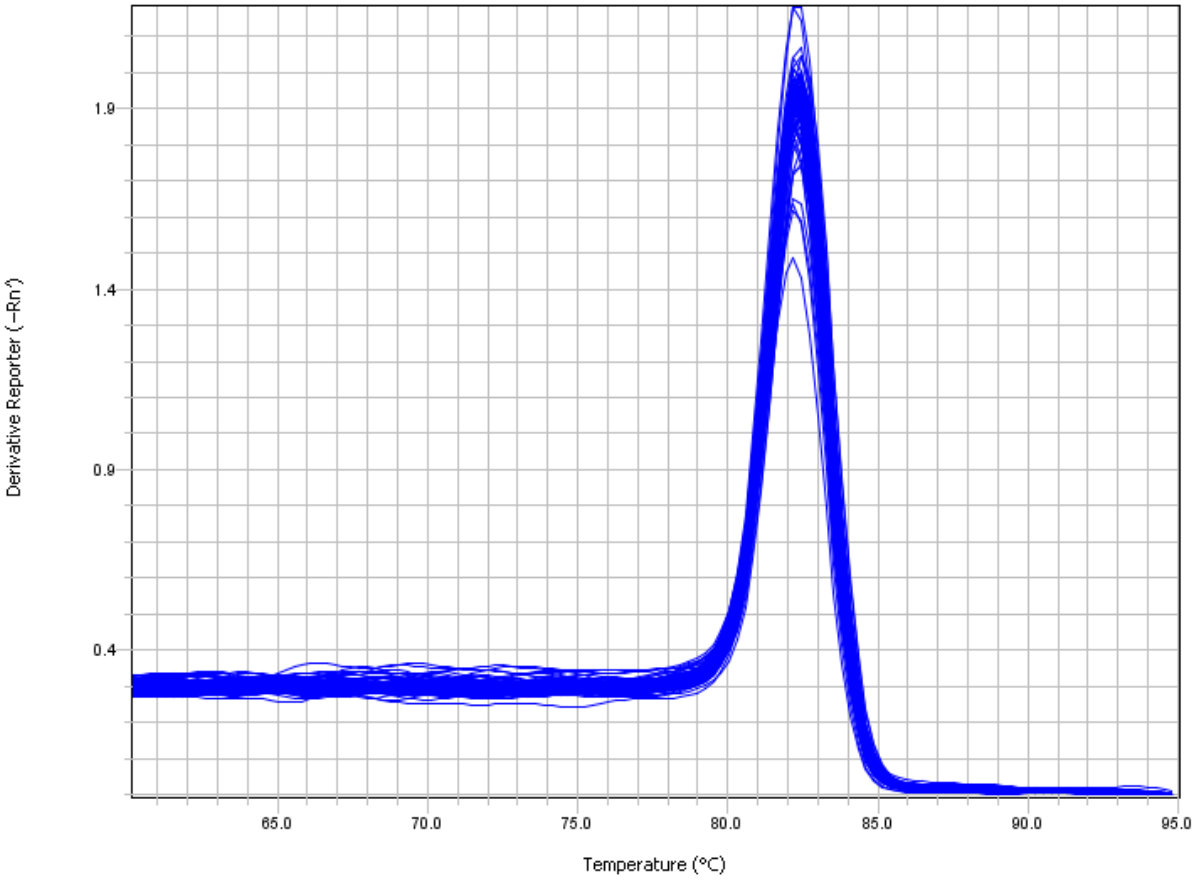

Figure 4.6 - *Example SYBR Green melt curve for GAPDH primers. Melt curve analysis performed to determine the specificity of each qPCR primer set. The generation of a distinct peak indicates the amplification of a single genomic region.*

PCR efficiency was determined from the slope of standards curve generated by plotting the cycle threshold against the log concentration of the 2-fold diluted samples (Figure 4.7). A linear regression was fitted to each standard curve, and the gradient of the slope was used to determine the efficiency of each mRNA expression assay. Efficiency estimates ranged between 95 and 105 % indicating highly efficient amplification of template in each assay. The efficiency of the reference gene *GAPDH* was 98.1% and the efficiencies of each gene of interest was within 10% (Figure 4.7; pooled gene of interest efficiency = 98.0% ± 3.27). A high coefficient of determination ($R^2 > 0.99$) was detected for the linear regression for each assay indicating linearity of amplification with increasing quantities of cDNA (Figure 4.7).

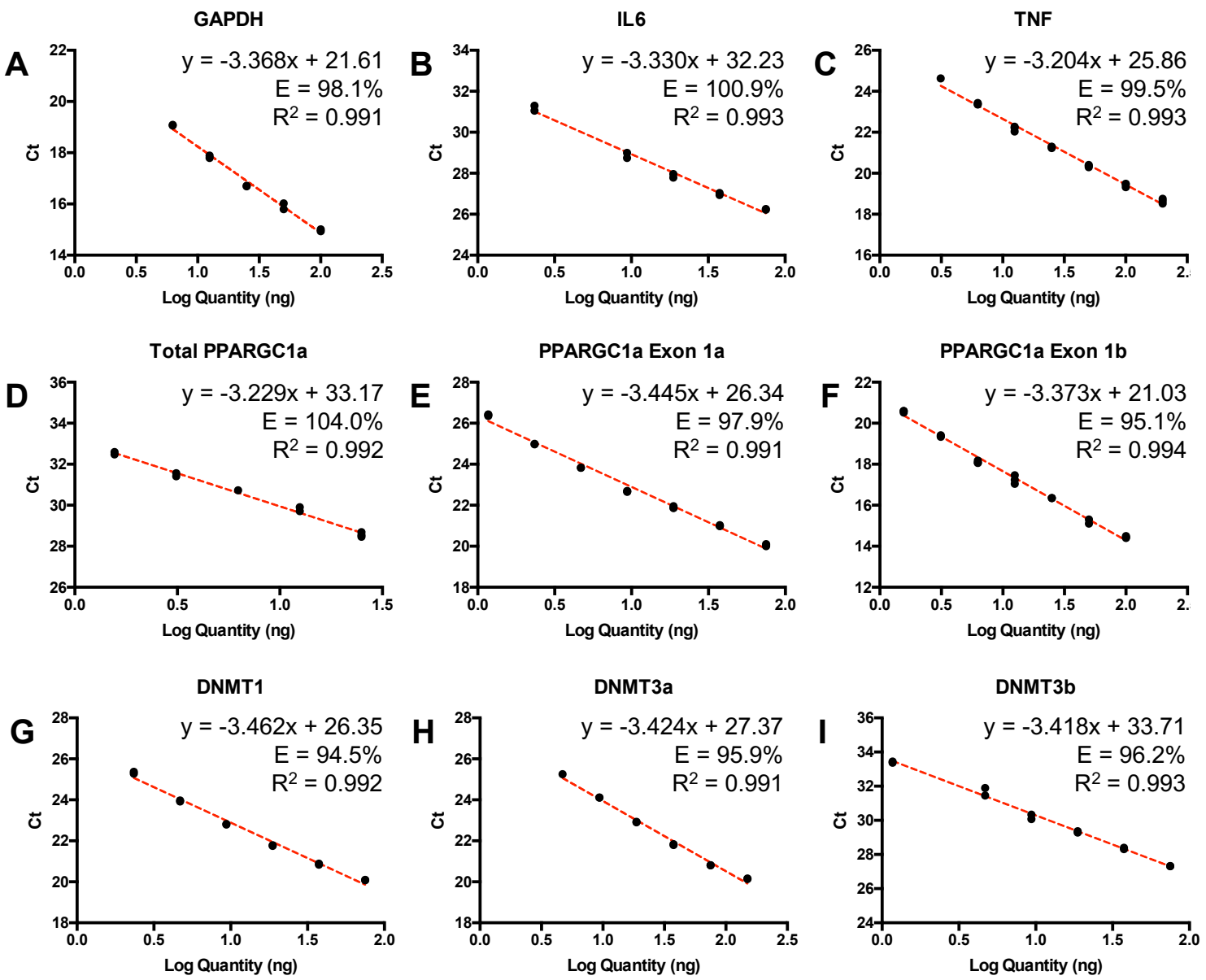

Figure 4.7 - *Validation of mRNA expression assays for (A) GAPDH, (B) IL6, (C) TNF , (D) Total PPARGC1A, (E) PPARGC1A Exon 1a, (F) PPARGC1A Exon 1b, (G) DNMT1, (H) DNMT3a and (I) DNMT3b. Red dashed line indicates linear regression curve between the log quantity of cDNA added to each reaction and the cycle threshold. Equation of the line, assay efficiency (E) and coefficient of determination (R^2) indicated in the top right of each graph.*

4.4 Interpretation

This chapter describes the validation of the assays used throughout this thesis for the determination of DNA methylation and mRNA expression. The accuracy and precision of DNA methylation, assessed by pyrosequencing, and mRNA expression, via RT-qPCR, relies on the specificity and efficiency of the assays used. Gel electrophoresis confirmed the specific amplification of the desired region of bcDNA and standard curves confirmed the absence of PCR bias. Melt curves confirmed the specific amplification of the desired template of mRNA and the linear and efficient amplification was confirmed using standard curves.

The generation of high-quality PCR product which is free of primer dimer and artefacts is one of the critical factors influencing accurate quantification (Noehammer et al. 2014). Each of the bcDNA standards produced a single band following gel electrophoresis (Figure 4.4) indicating a successful, high-quality PCR. The production of any off-target amplicons during PCR or remaining unincorporated primer following PCR can cause competitive binding between desired and undesired product and subsequently impact the determination of DNA methylation (Delaney et al. 2015).

Electrophoresis of PCR product also confirms the specificity of the pyrosequencing assays for bcDNA and the absence of amplification of gDNA (Figure 4.4). Amplification of non-converted DNA in the samples will falsely increase the detected DNA methylation because the non-methylated cytosines will remain as cytosine and be identified as 5mC during pyrosequencing (Genereux et al. 2009). The inclusion of a bisulfite conversion control in the dispensation order (Figure 4.3) provides a second quality control to ensure that contamination of samples with unconverted DNA does not impact the quantification of methylation (Tost and Gut 2007). The confirmation of assays which only amplify bcDNA and produce a single high-quality band of DNA following PCR confirm the specificity of the assays for the desired region.

The performance of each pyrosequencing assay was assessed for linear amplification of bcDNA of increasing methylation percentage to identify PCR bias caused by bisulfite conversion induced sequence differences between methylated and unmethylated DNA. PCR bias typically favours the amplification of unmethylated DNA

sequences leading to the underestimation of methylation (Wojdacz, Hansen, and Dobrovic 2008). At lowly methylated CpG sites, PCR bias can prevent the amplification of methylated DNA preventing the determination of any methylation. In the present study, assay performance was assessed using mixtures of commercially available unmethylated and methylated DNA to examine the relationship between input and observed methylation percentage.

The detected methylation was lower than the expected input methylation percentage for each of the standards other than the 0% methylated standard. Lower than expected DNA methylation percentage has previously been suggested to be caused by the incomplete methylation of control DNA by SssI methyltransferase (Murphy, Huang, and Hoyo 2012). Regardless of the lower than expected DNA methylation percentage, a linear increase in DNA methylation was detected with each of the assays. The high coefficient of determination ($R^2 > 0.99$) of the linear regression fitted to the standard curve of each assay indicates the absence of PCR bias (Figure 4.4).

The mRNA expression assays used in this thesis were selected from published sources (Campanelli et al. 2016; Korah et al. 2012; Popov et al. 2015; Silvennoinen et al. 2015; Wu et al. 2007) and should already have been validated for the determination of mRNA expression. The validation of the assays still had to be performed prior to their use in this thesis to determine mRNA expression for the target genes because assay performance is reliant on the experimental conditions used (Bustin and Huggett 2017). The RNA quality (Vermeulen et al. 2011) and both the reagents (Tesena et al. 2017) and thermocycler (Lu et al. 2010) used can all influence assay performance.

For the mRNA expression analysis in this thesis, assays have been selected with primers situated at exon junctions or spanning introns were selected to prevent any problems with DNA contamination. DNA contamination would not impact assays with primers situated at exon junctions because the inclusion of introns (present in DNA but not cDNA) removes primer binding sites from DNA sequences. Primers spanning introns would produce a PCR product if contaminated with DNA; however, a longer product would be produced because of the inclusion of the intron sequence which could be detected via melt curve analysis (Kuang et al. 2018; Wong and Medrano 2005). Primer-BLAST reports indicating the amplification of a single mRNA template

and the generation of a single melt peak for each assay indicate the assays to be used for mRNA analysis in the subsequent chapters are specific for the target region.

Melt curves were generated to ensure the specific amplification of a distinct product during qPCR (Figure 4.6). Similar to gel electrophoresis, which was used to confirm the specific high-quality amplification of the pyrosequencing methylation assays, melt curves can identify the generation of different sized PCR products. Unlike gel electrophoresis, melt curve analysis is also able to detect the generation of sequences with different GC percentages (Kuang et al. 2018; Ririe et al. 1997). A sequence with a GC percentage of 100% would produce a melt peak ~41 ºC greater than a sequence of the same length comprising of AT nucleotides. While short sequences (~40 bp which is indicative of primer dimer) would produce a melt peak ~12 ºC lower than a sequence of 1000 bp of similar GC percentage (Ririe et al. 1997).

The efficiency and linearity of PCR amplification are critical determinants of accurate quantification of mRNA expression. The slope and goodness of fit of the linear regression between decreasing C_t values with increasing input quantities of cDNA can be used to calculate reaction efficiency (Bustin and Huggett 2017; Wong and Medrano 2005). Efficiency estimates between 95–105 % and a linear standard curve indicated by an R^2 >0.99 indicate an optimised assay (Bustin and Huggett 2017). The high coefficient of determination indicates the goodness of fit of the linear regression; an R^2 > 0.99 signifies that 99% of the variation in fluorescence is explained by the increasing quantity of cDNA in the reaction. Each of the assays described above meet these validation criteria; therefore, can be used to investigate mRNA expression in the subsequent chapters of this thesis.

4.5 Conclusion

In conclusion, it is essential to validate the performance of assays to determine DNA methylation and mRNA expression to ensure precise and accurate quantification. Based on the criteria discussed above, each of the assays used in this thesis has successfully been validated against the methods to be utilised throughout this thesis. The successful validation of assay performance ensures that any change in DNA methylation or mRNA expression using these assays are the result of the interventions used and not the result of a poorly functioning assay. The next step is to use these assays to determine the impact of lifestyle interventions on DNA methylation and mRNA expression in the subsequent chapters of this thesis.

Chapter 5 - The impact of a bout of exercise to volitional fatigue and supplementation of n-3 PUFA on global and gene-specific DNA methylation.

5.1 Introduction

Several studies have demonstrated that exercise training interventions can modify the DNA methylome both at a global and gene-specific level (Denham et al. 2016; Denham, O'Brien, Harvey, et al. 2015; Denham, O'Brien, Marques, et al. 2015; Dimauro et al. 2016; Nitert et al. 2012; Rönn et al. 2013; Rowlands et al. 2014; Seaborne et al. 2018); however, less is known of the response of the DNA methylome to acute bouts of exercise. Acute exercise has been demonstrated to induce global hypomethylation in skeletal muscle (Barrès et al. 2012; Seaborne et al. 2018) and cell-free DNA located in plasma (da Silva et al. 2017); however, the global impact of acute exercise is unknown in leukocytes. The majority of the research surrounding the gene-specific response to acute exercise has focussed on the regulation of mitochondrial biogenesis (Bajpeyi et al. 2017; Barrès et al. 2012; Lane et al. 2015). The *PPARGC1A* gene, which encodes peroxisome proliferator-activated receptor gamma, co-activator alpha (PGC-1α) is considered to be the master regulator of mitochondrial biogenesis (Ventura-Clapier et al. 2008). Epigenetic studies have associated a CpG site -260 bases from the promoter of *PPARCG1A* with the regulation of mRNA expression. In skeletal muscle, exercise can demethylate the *PPARGC1A* -260 CpG site which has been shown to concurrently upregulate *PPARGC1A* mRNA expression (Bajpeyi et al. 2017; Barrès et al. 2012). *PPARGC1A* mRNA expression has also been demonstrated to be upregulated following acute exercise in leukocytes (Busquets-Cortés et al. 2017; Ferrer et al. 2009; Yakeu et al. 2010); however, it is unknown whether the methylation status of the -260 CpG site is critical in the regulation of mRNA expression in leukocytes.

The only previous study to investigate the impact of DNA methylation in leukocytes following acute exercise failed to identify any alteration in genome-wide DNA methylation following two-hours running on a treadmill interspersed with sprints every 10 min (Robson-Ansley et al. 2014). Despite no change in DNA methylation following exercise, incubating PBMCs with plasma taken from individuals who completed the same exercise protocol reduced the nuclear concentration of *DNMT3b* (Horsburgh et al. 2015). Reduced nuclear concentrations are likely the result of decreased mRNA expression which has previously been reported (Denham et al. 2016; Laye and

Pedersen 2010); however, it remains to be identified whether exercise-induced altered DNMT mRNA expression is sufficient to modulate DNA methylation patterns.

Although no change in leukocyte DNA methylation has been demonstrated following exercise, the exercise-induced increase in IL-6 concentrations displayed significant correlations with DNA methylation levels suggesting an epigenetic role for circulating lL-6 concentrations post-exercise (Robson-Ansley et al. 2014). The epigenetic role of IL-6 concentrations may be explained by the modulation of DNMT expression and DNA methylation patterns (Angelini et al. 2017; Foran et al. 2010; Hodge et al. 2001; Horsburgh et al. 2015). Despite the impact of IL-6 protein concentrations on DNA methylation, there is a sparsity of research on the DNA methylation of the *IL6* gene; however, leukocyte DNA methylation of *IL6* has previously associated with Rheumatoid Arthritis (Nile et al. 2008) and obesity (Na et al. 2015) indicating a role for *IL6* DNA methylation in the inflammatory response. Despite increased circulating levels of inflammatory cytokines post-exercise (Gjevestad et al. 2015; Gleeson et al. 2011), the impact of acute exercise on the DNA methylation of genes encoding inflammatory cytokines remains unknown.

Aside from exercise, other lifestyle interventions have been demonstrated to alter DNA methylation patterns (Alegría-Torres et al. 2011). Supplementation of the diet with n-3 PUFAs has been demonstrated to increase the content of n-3 PUFAs in whole blood (McGlory et al. 2014) and reduce levels of inflammation (Calder 2015; Rosignoli et al. 2013) and alter epigenome-wide DNA methylation (Amaral *et al.*, 2014; Hoile *et al.*, 2014; Karimi *et al.*, 2017; Tremblay *et al.*, 2017). Pathway analysis has demonstrated that inflammation and immune response are among the top regulated pathways (Tremblay et al. 2017). Gene-specific analysis has demonstrated a significant relationship between erythrocyte n-3 PUFA content and both *IL6* DNA methylation and IL-6 protein concentrations (Ma et al. 2016). The impact of n-3 PUFA supplementation on exercise-induced inflammation is inconclusive with some studies have reporting reductions in inflammation post-exercise with supplementation (Marques et al. 2015; Mickleborough et al. 2015), whereas, others have reported no change in inflammation (Martorell et al. 2014; Nieman et al. 2009). It remains to be identified whether the supplementation of n-3 PUFAs can modulate exercise-induced modification of DNA methylation patterns.

Aim

The primary aim of this chapter is to investigate the influence of a single bout of exercise on leukocytes DNA methylation and identify whether the response can be altered by the supplementation of n-3 PUFAs.

Objectives

1. Determine the impact of acute exercise and n-3 PUFA supplementation on global DNA methylation and mRNA expression of DNMT enzymes.
2. Identify any changes in DNA methylation or mRNA expression of *PPARGC1A* and *IL6* as a result of acute exercise or n-3 PUFA supplementation.
3. Determine if altered FA content of whole blood is associated with DNA methylation.

5.2 Methods

The experimental procedures for this study were approved by the Loughborough University Ethics Human Participants sub-committee (Study ID: R14-P185).

5.2.1 Participants

Ten healthy male participants were recruited into the study according to section 3.1. Participant characteristics are presented in Table 5.1.

Table 5.1- *Participant characteristics*

Variable	Mean ± SD (n = 10)
Age (yrs)	20.93 ± 1.06
Body Mass (kg)	75.30 ± 8.77
Height (m)	1.78 ± 0.07
BMI ($kg \cdot m^{-2}$)	23.58 ± 1.74
$\dot{V}O_{2peak}$ ($mL \cdot kg \cdot min^{-1}$)	57.19 ± 7.97

5.2.2 Study overview

The study consisted of a familiarisation session and two experimental trials. Each experimental trial was completed before (Before n-3PUFA) and after (After n-3PUFA) a two-week supplementation of n-3 PUFA (Figure 5.1A).

5.2.2.1 Familiarisation

Participants underwent anthropometric assessment for height and body mass (section 3.2.1). Peak oxygen uptake ($\dot{V}O_{2peak}$) was determined using a graded exercise test on a Lode Excalibur Sport electromagnetically braked ergometer (Lode B.V, Groningen, Netherlands). The exercise test began with an initial warm-up period of 4-min cycling at 100 W. Workload then increased to 130 W and increased by 35 W every 4-min until volitional fatigue was achieved (decrease in the self-selected cadence of 20 $revs \cdot min^{-1}$). Heart rate, Rating of Perceived Exertion (RPE; Borg 1982) and expired air were

collected in the final minute of each stage and when the participant perceived they only had one-minute remaining. Verbal encouragement was provided throughout the test.

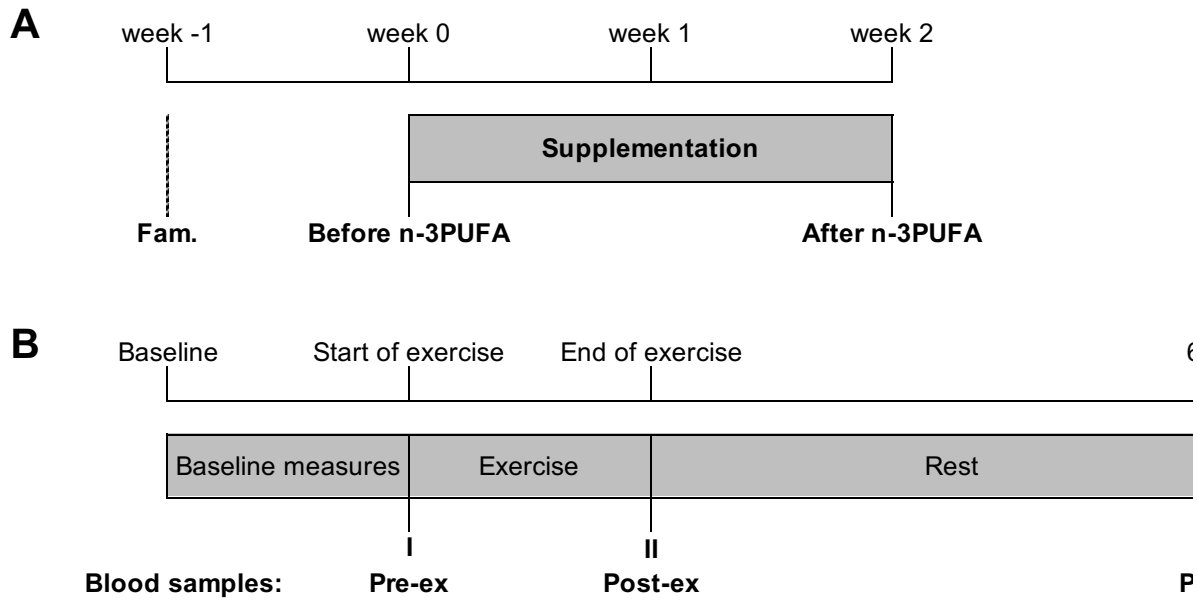

Figure 5.1 - *Schematic representation of (A) study outline and (B) trial day. Blood sampling performed at (I) Pre-ex, (II) Post-ex and (III) Post-ex+1hr in both the Before n-3PUFA and After n-3PUFA trials. Fam., familiarisation session; Before n-3PUFA, Before n-3 PUFA supplementation trial; After n-3PUFA, After n-3 PUFA supplementation trial; Pre-ex, pre-exercise; Post-ex, immediately post-exercise; Post-ex+1hr, 1-hour post-exercise.*

5.2.2.2 Experimental trials

Pre-trial standardisation of diet and exercise was performed according to section 3.2.2. Figure 5.1B provides a schematic representation of the trial day. On arrival to the laboratory, baseline measures were recorded (section 3.2.1) and participants rested in a seated position for 10 minutes prior to the collection of a baseline (Pre-ex) venous blood sample. Participants then completed the exercise bout consisting of a $\dot{V}O_{2peak}$ test (Figure 5.1B) using identical conditions as the familiarisation session. Further venous blood samples were collected immediately (maximum 2 minutes) following the cessation of exercise (Post-ex) and one-hour post-exercise (Post-ex+1hr). Participants remained within the laboratory in a seated position (Figure 5.1B; Rest) between the Post-ex and Post-ex+1hr blood samples.

5.2.2.3 Supplementation

The n-3 PUFA supplement (Holland and Barrett, UK) was provided to participants in capsule form. Participants were instructed to consume six capsules per day providing 5.7g of n-3 PUFA (4.08g of EPA and 1.62g of DHA) and 0.01g per day of α-Tocopherol. The n-3 PUFA dose was chosen based on previous findings showing that the dose was sufficient to induce changes in the FA profile of human blood (McGlory et al. 2014; Metherel et al. 2009). Capsule counts were used to determine the compliance of supplementation and confirmed using whole blood FA profiles.

5.2.3 Analytical procedures

5.2.3.1 Blood sampling

Venous blood samples were collected via venepuncture (section 3.3) at Pre-ex, Post-ex and Post-Ex+1hr during each trial for the assessment of DNA methylation, mRNA expression, whole blood cell counts and determination of the FA composition of whole blood.

5.2.3.2 DNA methylation

Genomic DNA was extracted from whole blood (section 3.4.1). The concentration of isolated gDNA was 177.87 (± 71.31) ng/uL with an A_{260}/A_{280} ratio of 1.91 (± 0.01). Global DNA methylation was assessed using LUMA (section 3.4.2).

For determination of gene-specific DNA methylation, DNA was bisulfite-converted (section 3.4.1) and underwent PCR using the PyroMark PCR kit. DNA methylation percentage was determined via pyrosequencing (section 3.4.3). The assays used to determine DNA methylation are presented in Table 4.2 (PPARGC1A and IL6).

5.2.3.4 Analysis of mRNA expression

RNA was extracted from whole blood. The concentration of isolated RNA was 134.10 (± 57.69) ng/uL and an A_{260}/A_{280} ratio of 2.09 (± 0.01). RNA was then cDNA converted (section 3.4.5), and relative mRNA expression was performed using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001) using GAPDH as the reference gene (section 3.4.6). Primer sequences for the assays used to determine mRNA expression of the genes of interest are displayed in Table 4.3 (*GAPDH, Total PPARGC1A, IL6, DNMT1,*

DNMT3a and DNMT3b). The mean Ct value of GAPDH across all participants and experimental conditions was 16.839 (±0.45) with a low variation of 2.68%.

5.2.3.5 Fatty acid composition of whole blood

Fatty acid methyl esters (FAME) were prepared via incubating 100 µL of whole blood with 3.4 mL of 50 µg/mL BHT-methanol, 200µL of acetyl chloride and 100 µL of 10 µg/mL of heptadecanoic acid (to act as an internal standard) at 70°C for 60 minutes. The reaction was cooled, and 5 mL of 6% (K_2CO_3) was added to stop and neutralise the reaction. The sample was washed by adding 1.5 mL of hexane and centrifuged at 2500 RPM for 10 minutes. The top layer of hexane was removed, and the washing step was repeated. Both hexane layers were combined and evaporated until dry using nitrogen gas. When dry, the sample was reconstituted in 100 µL of hexane and frozen at -20ºC until further analysis.

The sample analysis involved the use of an Agilent Technologies 7820A GC system (Agilent Technologies, USA) fitted to an Agilent Technologies 5977B MSD sporting a single quadrupole mass analyser (Agilent Technologies, USA) and a non-polar DB-5ms (30m x 0.25mm internal diameter x 0.25µm film thickness) column (Agilent Technologies, USA). A 1 µL sample was injected on the GC-MS with the oven temperature program set at an initial temperature of 130ºC and then increased from 130ºC to 208ºC at a rate of 6ºC/min. The temperature was programmed to increase at a rate of 2 ºC/min to 225ºC where it was held for 10 min followed by a final ramp of 25 ºC/min to 300 ºC and held for a final minute (total analysis time = 35.5 min). The injector temperature was set at 230ºC with a splitless flow of 100 mL/min at 1 minute. Helium was used as carrier gas with a constant flow rate of 1.5 mL/min. The MSD transfer line was set at 230ºC, the ion source temperature was set at 230ºC and the MS Quad was set at 150ºC. FAMEs were characterised using electron ionisation (EI) in full scan mode (m/z 40 - 400) at a scan rate of 5.9 scans/second. The individual FAMEs were identified by comparing to the retention times of a Supelco 37 Mix FAME standard (Sigma-Aldrich, USA) which can be used to identify complex mixtures of saturated, monounsaturated and polyunsaturated complemented with the MS NIST library. Mass Hunter Qualitative Analysis Navigator (Agilent Technologies, USA) and MS Quantitative Analysis software (Agilent Technologies, USA) were used in the

qualification and quantitation of the FAMEs present respectfully. The results were expressed as the relative percentages of the total identified FA.

5.2.4 Statistical Analysis

Statistical analysis was performed according to section 3.5. The change in the FA composition of whole blood was detected using paired t-tests (Before n-3PUFA vs After n-3PUFA). DNA methylation and mRNA expression values were analysed using a 2 (trial) x 3 (time) RM-ANOVA. Spearman's Rho correlation analysis was used to assess the relationship between DNA methylation and whole blood FA profiles according to section 3.5. All data presented as mean ± 95% CI unless otherwise stated.

5.3 Results

5.3.1 Whole blood fatty acid composition

Whole blood FA profiles are presented in Table 5.2. Following n-3 PUFA supplementation, the total percentage of the n-3 PUFAs EPA, DHA and DPA increased (Figure 5.2); however, there was no change in the percentage of ALA ($p > 0.05$; Figure 5.2E). Alongside the increased percentage of n-3 PUFAs, the relative percentage of n-6 PUFA ($p = 0.040$) reduced (Table 5.2). The opposing impact of n-3 PUFA supplementation on the relative percentage of n-3 and n-6 PUFAs caused a reduction in the n-6 PUFA / n-3 PUFA ratio (Figure 5.2B).

Table 5.2 – *Pre-ex whole blood fatty acid profiles. Values indicate mean percentage of total fatty acids within whole blood ± 95% confidence intervals. Significant change in composition of whole blood indicated by * ($p < 0.05$) or # ($p < 0.01$).*

Common Name	Shorthand Notation	Before n-3PUFA	After n-3PUFA
Myristic acid	14:0	1.29 ± 0.34	0.92 ± 0.23
Pentadecanoic acid	15:0	0.26 ± 0.04	0.22 ± 0.04
Palmitic acid	16:0	25.75 ± 1.27	23.55 ± 1.37
Stearic acid	18:0	8.1 ± 0.79	7.7 ± 0.45
Behenic acid	22:0	0.18 ± 0.04	0.19 ± 0.02
Lignoceric acid	24:0	0.5 ± 0.07	0.48 ± 0.09
Total Saturated		**36.08 ± 1.67**	**33.06 ± 1.77#**
Palmitoleic acid	16:1 n-7	0.69 ± 0.24	0.44 ± 0.13
Oleic acid	18:1 n-9	9.53 ± 1.11	8.08 ± 1.03
Total Monounsaturated		**10.22 ± 1.35**	**8.51 ± 1.16***
Linoleic acid (LA)	18:2 n-6	28.23 ± 1.28	27.09 ± 1.55
Gamma-linoleic acid	18:3 n-6	0.4 ± 0.1	0.26 ± 0.07
Arachidonic acid (AA)	20:4 n-6	14.15 ± 1.27	13.64 ± 0.78
Adrenic acid	22:4 n-6	1.58 ± 0.22	1.46 ± 0.14
Total n-6 PUFA		**44.36 ± 1.54**	**42.45 ± 1.68***
Alpha-linolenic acid (ALA)	18:3 n-3	1.34 ± 0.54	1.38 ± 0.67
Eicosapentaenoic acid (EPA)	20:5 n-3	1.67 ± 0.47	6.36 ± 0.83
Docosapentaenoic acid (DPA)	22:5 n-3	1.6 ± 0.24	2.31 ± 0.3
Docosahexaenoic acid (DHA)	22:6 n-3	4.73 ± 1.05	5.93 ± 0.96
Total n-3 PUFA		**9.35 ± 1.31**	**15.97 ± 1.17#**

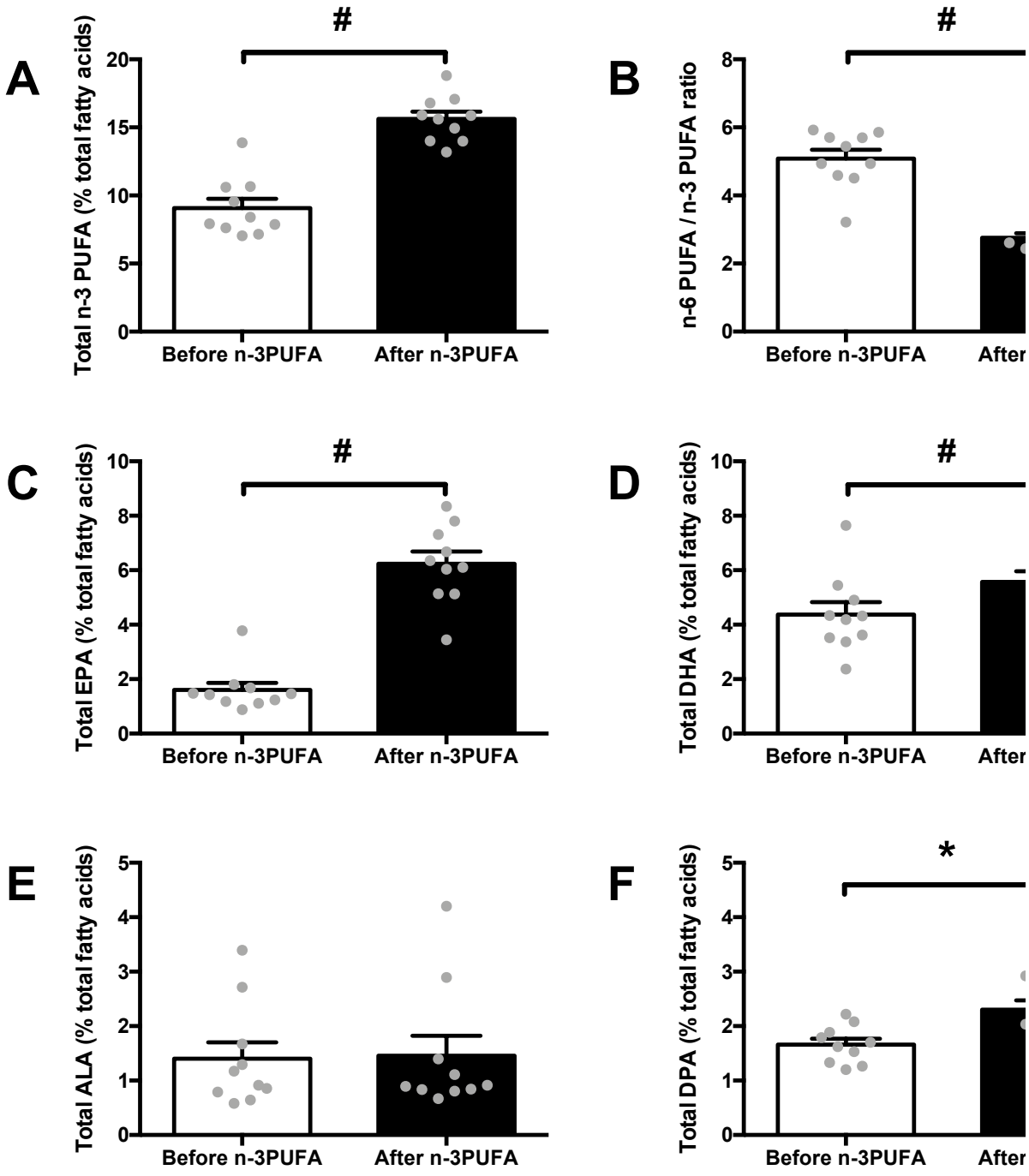

Figure 5.2 - *Whole blood fatty acid profile for (A) total n-3 polyunsaturated fatty acid (PUFA), (B) n-6 PUFA / n-3 PUFA ratio, (C) total eicosapentaenoic acid (EPA), (D) total docosahexaenoic acid (DHA), (E) total alpha-Linolenic acid (ALA) and (F) total docosapentaenoic acid (DPA) expressed as percentage of total fatty acids. Data presented as mean ± 95% confidence intervals. Grey circles indicate individual data points. * p < 0.05; # p < 0.01.*

5.3.2 Global DNA methylation and DNMT mRNA expression

There was no impact of exercise or n-3 PUFA supplementation on global DNA methylation (Figure 5.3A); however, a significant reduction in DNMT mRNA expression was detected following exercise. Post-ex *DNMT3a* mRNA expression was reduced ($p < 0.001$) and returned to Pre-ex values by Post-ex+1hr ($p > 0.05$; Figure 5.3C), whereas, *DNMT1* ($p = 0.004$; Figure 5.3B) and *DNMT3b* ($p = 0.036$; Figure 5.3D) were significantly reduced at Post-ex+1hr.

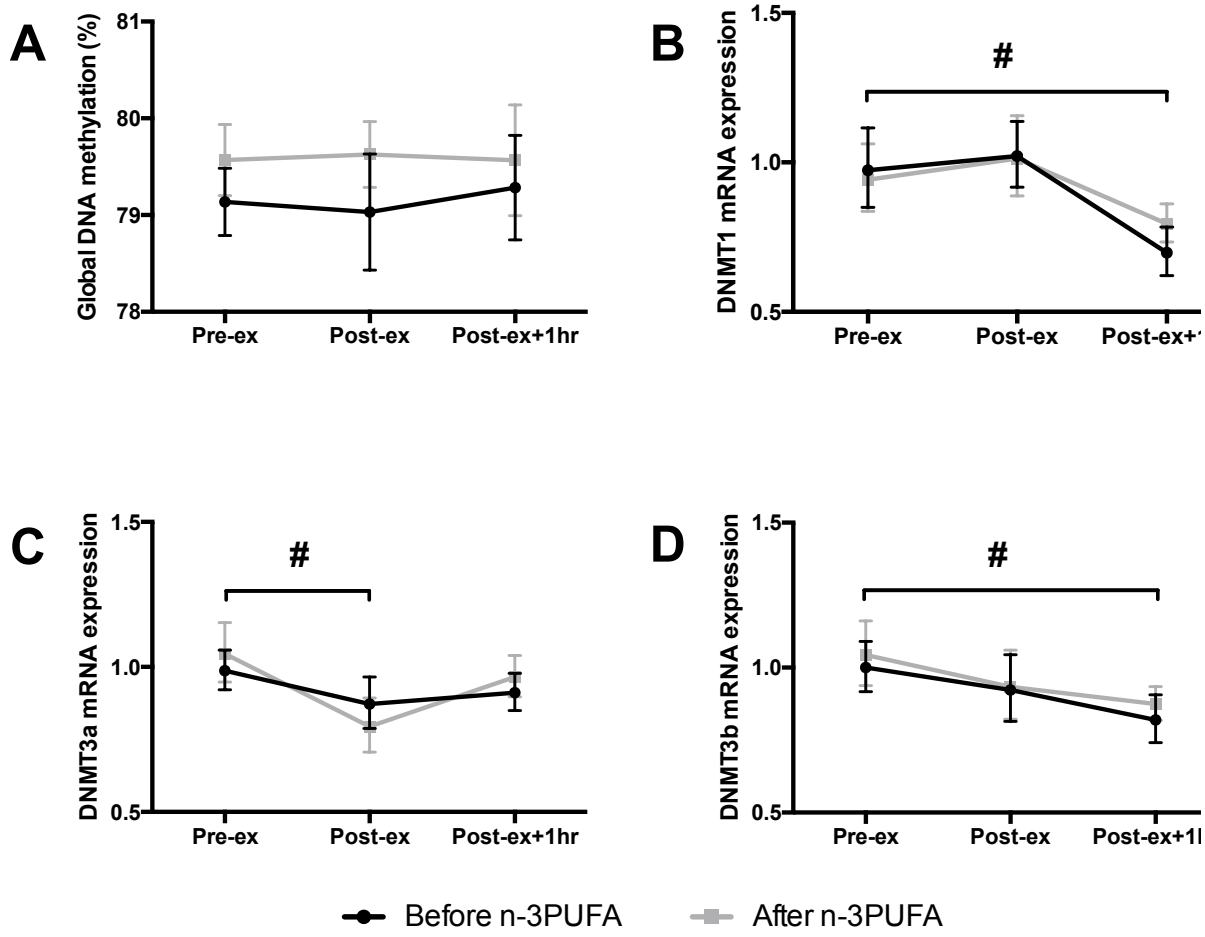

Figure 5.3 - *Effect of exercise and n-3 PUFA supplementation on (A) global DNA methylation and the mRNA expression of (B) DNMT1, (C) DNMT3a and (D) and DNMT3b. Significant impact of time indicated by * ($p < 0.05$) or # ($p < 0.01$).*

5.3.3 Gene-specific DNA methylation and mRNA expression

5.3.3.1 PPARGC1A

Neither exercise or supplementation altered *PPARGC1A* DNA methylation (Figure 5.4A). A main effect was detected for time with *PPARGC1A* mRNA expression (p = 0.027) indicating an increase in *PPARGC1A* expression with exercise; however, this failed to remain significant following correction for multiple comparisons, and only a trend existed for increased mRNA expression Post-ex+1hr (p = 0.058) compared to Pre-ex (Figure 5.4B).

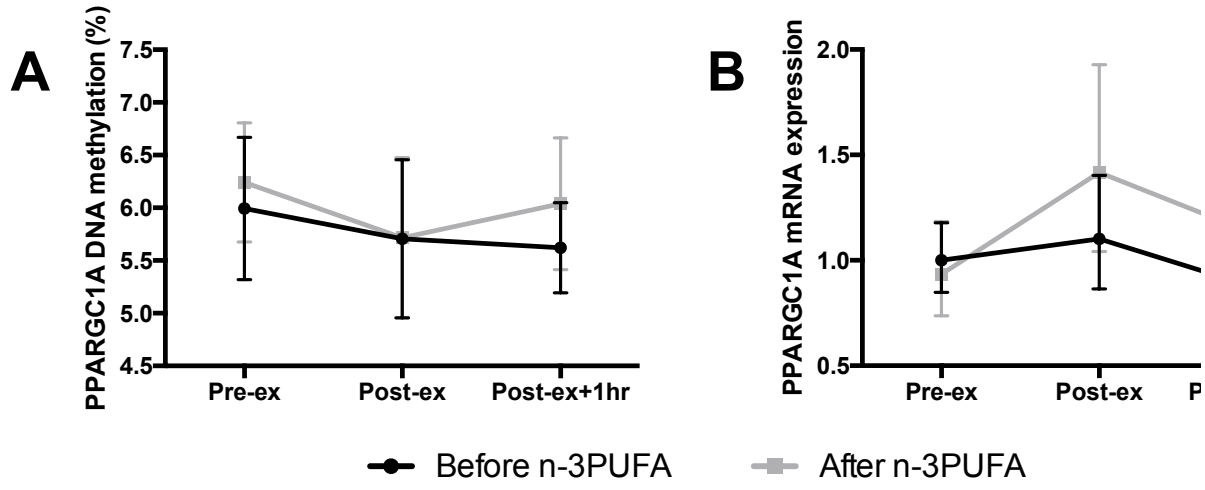

Figure 5.4 - *Effect of exercise and supplementation on (A) the DNA methylation of a single CpG site -260 bases from the TSS of the PPARGC1A promoter and (B) mRNA expression of PPARGC1A. Significant impact of time indicated by * (p <0.05) or # (p <0.01).*

5.3.3.2 IL6

Pre-ex, supplementation had no impact on DNA methylation or mRNA expression (p > 0.05). Immediately post-exercise, the DNA methylation of *IL6* CpG2 was significantly reduced After n-3PUFA compared to Before n-3PUFA (p = 0.004; Figure 5.5A). A trend was detected for increased *IL6* mRNA expression (p = 0.077) Post-ex which became significant at Post-ex+1hr (p < 0.001; Figure 5.5B) in the After n-3PUFA trial compared to Before n-3PUFA. Neither exercise or the n-3 PUFA supplementation altered DNA methylation of any of the other *IL6* CpG sites (p > 0.05). *IL6* DNA methylation was uncorrelated to *IL6* mRNA expression (p > 0.05).

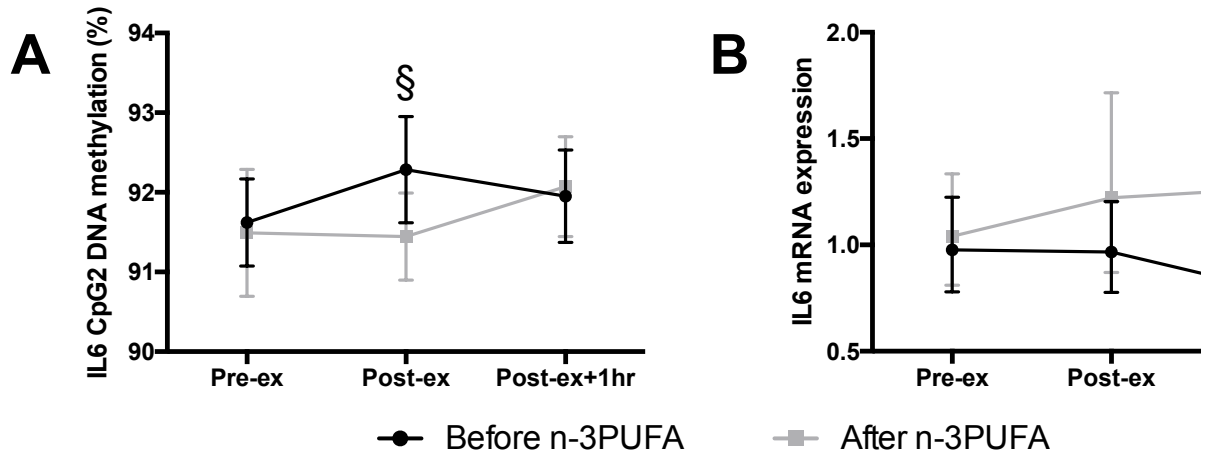

Figure 5.5 - *Effect of exercise and supplementation on (A) the DNA methylation of a single CpG site -1096 bases (CpG2) from the TSS of the IL6 promoter and (B) mRNA expression of IL6. A significant interaction between supplementation and time indicated by § ($p < 0.01$).*

5.3.4 Impact of whole blood fatty acid profile on DNA methylation

Following n-3 PUFA supplementation, significant positive correlations were detected between the total percentage of n-3 PUFAs and *IL6* DNA methylation Post-ex (CpG2 $p = 0.008$; CpG5 $p = 0.006$; CpG Avg $p = 0.004$; Figure 5.6), whereas, significant negative correlations were detected with the n-6 PUFA / n-3 PUFA ratio and *IL6* DNA methylation (CpG2 $p = 0.033$; CpG5 $p = 0.029$; CpG Avg $p = 0.022$; Figure 5.6). No other associations were detected between DNA methylation and the percentage of saturated, monosaturated, n-6 PUFA or n-3 PUFAs either before or after n-3 PUFA supplementation.

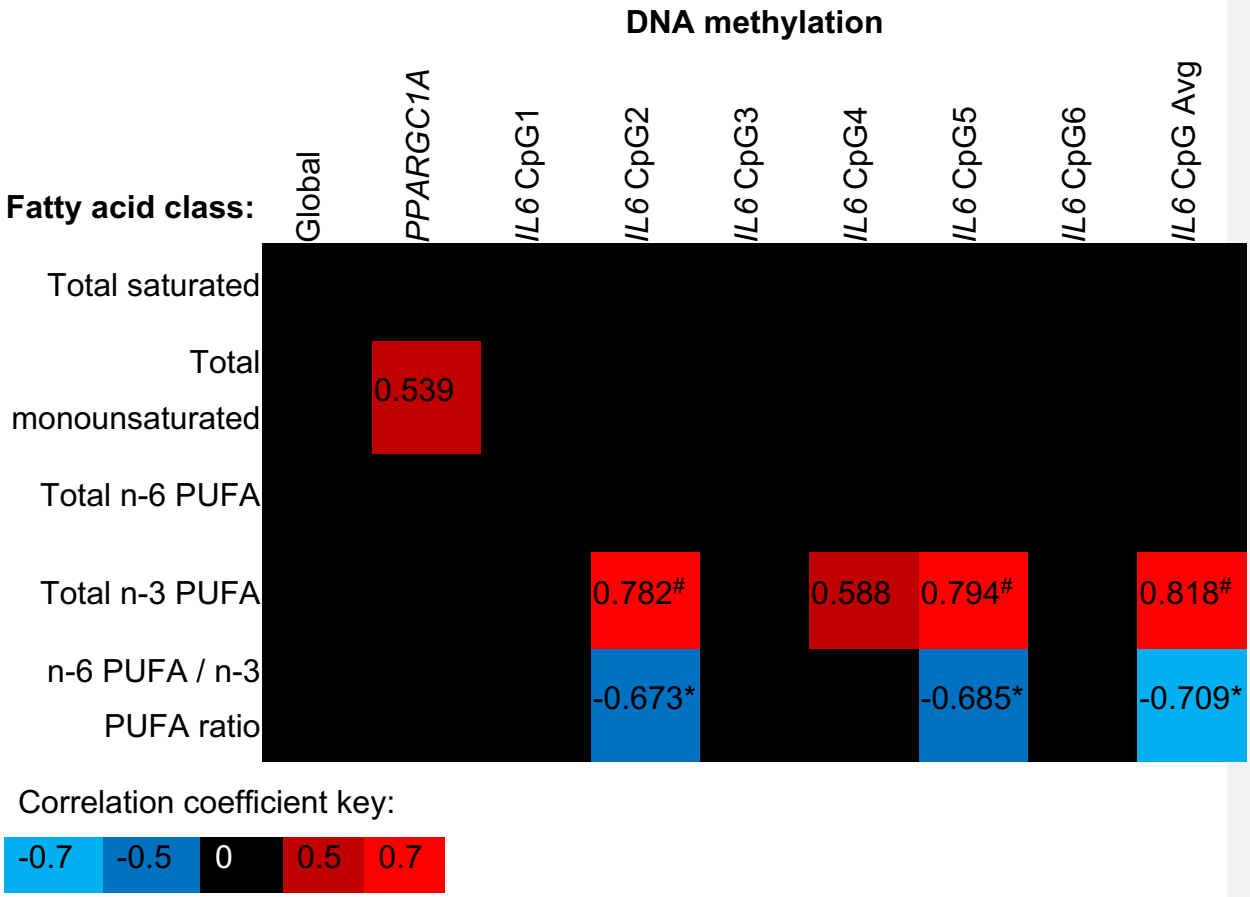

Figure 5.6 – *Spearman's Rho between after n-3PUFA supplementation DNA methylation and fatty acid class (percentage of total fatty acids). Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5 (see key). *p < 0.05, # p < 0.01.*

5.3.5 Association between DNA methylation and exercise performance

No correlations were identified between global DNA methylation and measures of exercise performance either before or after the supplementation of n-3 PUFAs ($p > 0.05$; Figure 5.7). A significant positive correlation was identified between Post-ex+1hr PPARGC1A DNA methylation and $\dot{V}O_{2peak}$ before n-3 PUFA supplementation ($p = 0.038$; Figure 5.7); however, this association disappeared following n-3 PUFA supplementation (Figure 5.7). Post-ex DNA methylation of IL6 CpG 2 was negatively correlated to $\dot{V}O_{2peak}$ both before and after n-3PUFA supplementation ($p < 0.05$; Figure 5.7); whereas, Post-ex+1hr significant negative correlations between exercise performance and DNA methylation of IL6 CpG2 were detected only after n-3 PUFA supplementation ($p < 0.05$; Figure 5.7). None of the other IL6 CpG sites investigated (or the mean methylation) were associated with exercise performance ($p > 0.05$).

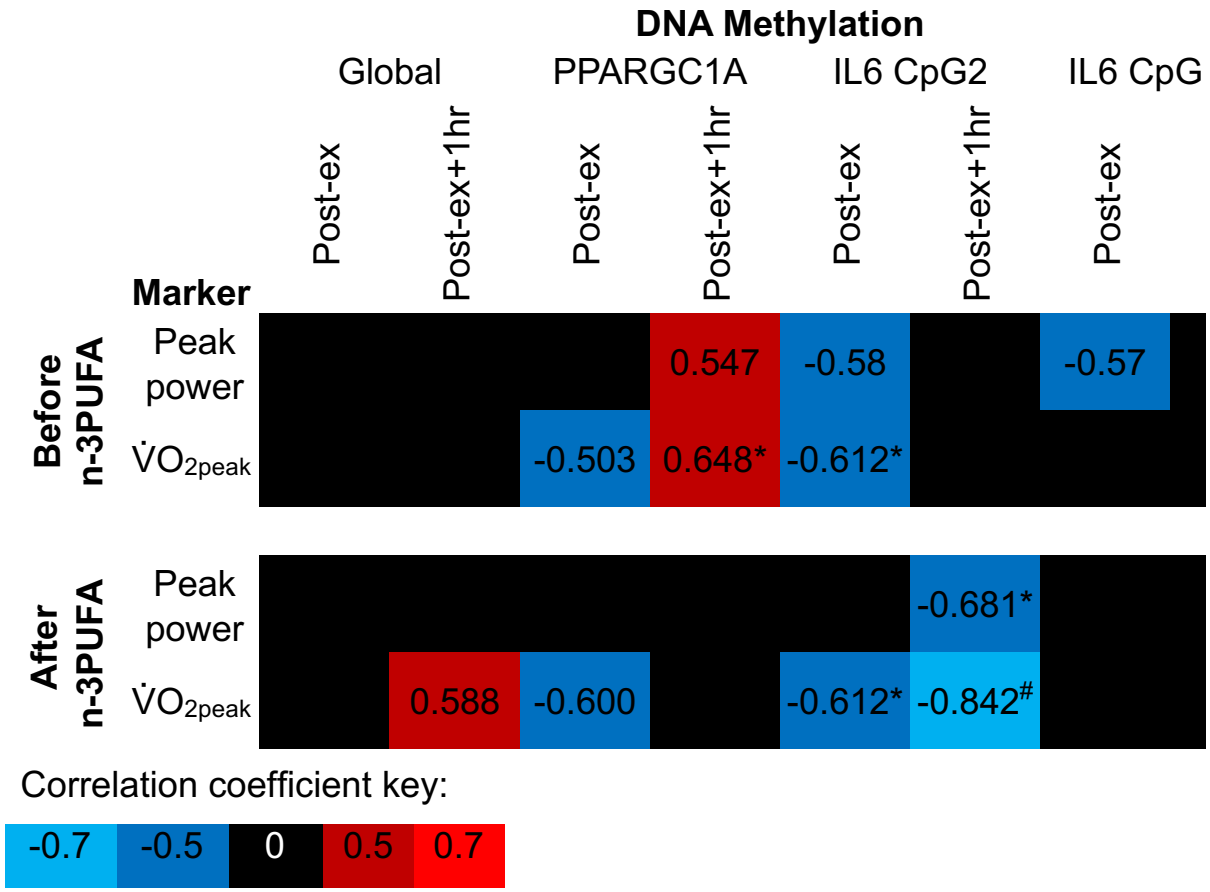

Figure 5.7 - *Spearman's Rho between post-exercise DNA methylation and measures of exercise performance. Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. Peak power, peak power achieved during exercise test; $\dot{V}O_{2peak}$, peak oxygen uptake. *p < 0.05, # p < 0.01.*

5.4 Discussion

This chapter aimed to investigate the impact of a single bout of exercise to volitional fatigue before and after a 2-week supplementation of n-3 PUFAs on global and gene-specific DNA methylation and mRNA expression. Global DNA methylation was unchanged following exercise and supplementation; however, DNMT mRNA expression was reduced following exercise suggesting a potential role for exercise altering DNA methylation. The supplementation of n-3 PUFAs did not alter the DNA methylation of *IL6*; however, significant correlations were detected between *IL6* DNA methylation and the proportion of n-3 PUFAs and the n-6 / n-3 PUFA ratio of whole blood. Although neither exercise or n-3 PUFA supplementation altered DNA methylation, an interaction was detected between exercise and supplementation for *IL6* DNA methylation and mRNA expression. Immediately post-exercise, the DNA methylation of a single site of *IL6* was significantly reduced following n-3 PUFA supplementation compared with pre-supplementation. Although not significant a trend for increased *IL6* mRNA expression was detected at the same time point and this association became significant 1 hr post-exercise.

The lack of association between acute exercise and global DNA methylation in the present study is contrary to previous reports suggesting that an acute bout of exercise is sufficient to reduce global methylation (Barrès et al. 2012; da Silva et al. 2017). A similar exercise protocol to volitional fatigue reduced global DNA in the skeletal muscle of sedentary adults (n = 14) (Barrès et al. 2012), whereas, 90-min of treadmill walking reduced methylation in the plasma of chronic obstructive pulmonary disease (COPD) patients (n = 13) (da Silva et al. 2017). The only previous study to investigate the impact of acute exercise in leukocytes failed to detect altered genome-wide DNA methylation (Robson-Ansley et al. 2014). These results suggest a potential tissue-specificity between skeletal muscle and leukocytes for the response of DNA methylation to acute exercise. A tissue-specific response to exercise training has been demonstrated by the alteration of different molecular pathways in skeletal muscle (Nitert et al. 2012) and adipose tissue (Rönn et al. 2013) of the same individuals following a six-month exercise training intervention.

Alternatively, the differences in global methylation between studies may be explained by the exercise capacity of the participants. The $\dot{V}O_{2peak}$ of the participants in the present study is similar to the $\dot{V}O_{2max}$ reported by Robson-Ansley et al.,(2014) (57 vs 53 mL·kg·min^{-1}); whereas, lower exercise capacities are reported in the cohort of sedentary individuals (42 mL·kg·min^{-1}) and estimated in the COPD patients (16 mL·kg·min^{-1}; estimated using the equation reported by Ross et al. (2010) using the 6-minute walk distance) (Barrès et al. 2012; da Silva et al. 2017). The impact of baseline fitness on the DNA methylation response to exercise is unknown; however, global hypomethylation is consistently reported with exercise training indicating that regular exercise reduces global methylation (Denham, O'Brien, Marques, et al. 2015; Dimauro et al. 2016; Nitert et al. 2012; Rowlands et al. 2014; Seaborne et al. 2018). The $\dot{V}O_{2peak}$ of the participants in the current study indicates a high level of fitness (within the top quartile (Kaminsky, Arena, and Myers 2015)); therefore, the hypomethylation induced by exercise training may have already occurred making the exercise stimulus insufficient to stimulate further hypomethylation.

In the present study, the supplementation of n-3 PUFAs did not alter global DNA methylation. Although there is little evidence on the impact of n-3 PUFA supplementation on global DNA methylation, a significant reduction in LINE-1 methylation has been reported following a 6-month n-3 PUFA supplementation in Alzheimer's patients (Karimi et al. 2017). *LINE-1* methylation is reported to be increased in Alzheimer's patients compared to healthy controls (Di Francesco et al. 2015); therefore, the supplementation of n-3 PUFA in these individuals may act to restore global DNA methylation to the normal level detected in healthy individuals explaining the lack of association within the present cohort of healthy individuals. Aside from the participants, the use of different surrogate measures of global methylation (LUMA vs *LINE-1*) prevents the direct comparison between studies, two separate studies have indicated that the methylation estimates provided by LINE-1 and LUMA are poorly correlated (Lisanti et al. 2013; Wu et al. 2011), indicating that changes in methylation detected by one method may not be detectable when using the other.

Despite a lack of change in global DNA methylation following exercise and n-3 PUFA supplementation in the present study, it cannot be concluded that the interventions did not have a significant impact on the methylome. Exercise-training EWAS have

reported differential methylation across thousands of CpG sites; while some studies report a large proportion of CpG sites display reduced methylation (Denham, O'Brien, Marques, et al. 2015; Nitert et al. 2012; Rowlands et al. 2014; Seaborne et al. 2018), others reported similar numbers of CpG sites increasing and decreasing in DNA methylation (Denham et al. 2016; Lindholm et al. 2015). When the directionality of global methylation changes was investigated using LUMA, one study detected no significant change in the average methylation post-training compared to pre-training despite differential DNA methylation being detected in ~4000 CpG sites (Lindholm et al. 2015). These data indicate the importance of examining gene-specific DNA methylation alongside measures of global DNA methylation to examine the impact of interventions.

The DNMT enzymes are known to play a vital role in controlling DNA methylation, in the present study we examined the mRNA expression of *DNMT1*, *DNMT3a* and *DNMT3b* to examine whether exercise and n-3 PUFA supplementation were sufficient to alter the expression. Exercise, but not the supplementation of n-3 PUFAs, was sufficient to alter the mRNA expression of each of the DNMTs. Previously, only the mRNA expression of *DNMT3b* has been demonstrated to be altered following acute exercise (Laye and Pedersen 2010) or a training intervention (Denham et al. 2016). The only other association between DNMTs and exercise in humans reported decreased nuclear concentrations of *DNMT3b* in PBMCs incubated with exercise conditioned plasma (Horsburgh et al. 2015). Although these studies suggest that the altered expression/concentration of *DNMT3b* may modify DNA methylation, there is a lack of evidence to support this conclusion. Aside from exercise, disease studies have reported positive relationships between the mRNA expression of DNMTs and DNA methylation (Jaiswal et al. 2015; Kobayashi et al. 2011). Therefore, despite no change in global DNA methylation in the present study, the decreased mRNA expression of DNMTs suggests that an acute bout of exercise may alter the DNA methylation in leukocytes.

In the present study, we did not detect altered DNA methylation or mRNA expression of *PPARGC1A* following either acute exercise or n-3 PUFA supplementation. Previously, within skeletal muscle, exercise has been sufficient to decrease the DNA methylation of same CpG site of the PPARGC1A promoter (Bajpeyi et al. 2017; Barrès

et al. 2012; Nitert et al. 2012), whereas high fat overfeeding studies have been associated with increased *PPARGC1A* DNA methylation in both skeletal muscle and adipose tissue (Gillberg et al. 2014; Perfilyev et al. 2017). There is a lack of previous literature on the impact of lifestyle interventions on the methylation status of the -260 CpG site of the PPARGC1A promoter; therefore, it is unknown whether the CpG site regulates *PPARGC1A* mRNA expression in leukocytes or if the interventions used in the present study were insufficient to alter the methylation.

Although no change in PPARGC1A methylation was identified following acute exercise in the present study, a positive correlation was identified between Post-ex+1hr PPARGC1A and the $\dot{V}O_{2peak}$ during the exercise test. These results potentially suggest that individuals with higher fitness do not undergo a hypomethylation response following acute exercise because the exercise bout provided an insufficient stimuli to induce adaption. The only previous study to investigate the association between PPARGC1A methylation and measures of fitness failed to find an association between the amount of moderate-vigorous physical activity (mins/day) and leukocyte DNA methylation at a region further upstream (-841 to -515 bp) of the PPARGC1A promoter (Clarke-Harris et al. 2014). The lack of clarity between the studies may result from the use of a different region of the PPARGC1A promoter in the study by Clarke-Harris (2014), which has not previously associated with exercise.

The inflammatory response to a bout of exercise has been demonstrated to be critical in altering DNA methylation in leukocytes, Robson-Ansley et al., (2014) reported significant associations between plasma IL-6 concentrations and DNA methylation of 53 CpG sites. Exercise-induced increased *PPARGC1A* mRNA expression in leukocytes has been suggested to induce an anti-inflammatory phenotype (Busquets-Cortés et al. 2017; Yakeu et al. 2010) and alter the anti-oxidant defence in leukocytes (Ferrer et al. 2009). The lack of increase in *PPARGC1A* mRNA expression following exercise in the current study suggests the exercise may have been insufficient to alter the anti-inflammatory and anti-oxidant defence, and subsequently DNA methylation.

In the present study, no assessment of circulating IL-6 concentration was determined; however, the impact of exercise and n-3 PUFA supplementation on *IL6* DNA methylation and mRNA expression was investigated. A region ~1000 bp upstream of

the TSS of the *IL6* promoter spanning six CpG sites (-1099 to -1057) was selected to examine DNA methylation because this region regulates *IL6* mRNA expression (Nile et al. 2008) and associates with both obesity (Na et al. 2015), and rheumatoid arthritis (Nile et al. 2008). Following n-3 PUFA supplementation, significant positive correlations were detected between *IL6* DNA methylation and whole blood n-3 PUFA content and the n-6 PUFA / n-3 PUFA ratio (Figure 5.6); however, this association did not result in altered DNA methylation. A significant negative relationship has previously been reported between the duration of n-3 PUFA supplementation and IL-6 concentrations (Li et al. 2014), potentially indicating the supplementation duration used in the present study may be insufficient to alter DNA methylation and mRNA expression.

The only previous report of the impact of n-3 PUFA supplementation on *IL6* DNA methylation detected an opposing relationship between n-3 PUFA content and *IL6* DNA methylation. Ma *et al.*, (2016) detected higher erythrocyte n-3 PUFA content was associated with decreased *IL6* DNA methylation for a single CpG site in the region of the *IL6* promoter closer to the TSS. Although a positive correlation was also detected between n-3 PUFA content and IL-6 protein concentrations, the observational nature of the study prevents the conclusion that the mechanism via which n-3 PUFAs alter *IL6* protein concentrations is *IL6* DNA methylation. The opposing relationship between n-3 PUFA content and *IL6* DNA methylation may be explained by the use of different cell populations (erythrocytes vs whole blood) for the determination of fatty acid content and the use of CpG sites in different regions of the *IL6* promoter.

In the present work, immediately post-exercise the DNA methylation of a single *IL6* CpG site (CpG 2) was detected to be significantly lower following n-3 PUFA supplementation compared to the trial before n-3 PUFA supplementation. In accordance with these findings, the present work identified negative correlations between DNA methylation of the same IL6 CpG site and both peak power and $\dot{V}O_{2peak}$ during the exercise, with the strongest correlations following supplementation of n-3 PUFAs. Alongside reduced DNA methylation a trend existed for increased mRNA expression existed immediately post-exercise following n-3 PUFA supplementation which became significant one-hour post-exercise.

Although the results appear to suggest that the supplementation of n-3 PUFAs increase the level of inflammation following an acute bout of exercise, this may not be true. Although exercise training is known to reduce systemic IL-6 concentrations (Christiansen et al. 2010; Kim 2014; Oberbach et al. 2008), increased *IL6* mRNA expression has been in reported leukocytes of highly trained athletes compared to lowly individuals and sedentary controls (Capomaccio et al. 2011). Unlike other inflammatory cytokines, *IL6* is known to possess pleiotropic roles within the inflammatory response (Scheller et al. 2011). Chronic expression of *IL6* or increased IL-6 in response to infection is associated with increased expression of pro-inflammatory cytokines (Petersen and Pedersen 2006), whereas, the transient-exercise-induced elevations in IL-6 has been demonstrated to possess anti-inflammatory roles via the expression of IL-1ra and IL-10 (Steensberg et al. 2003) and the inhibition of *TNF* (Gleeson et al. 2011; Petersen and Pedersen 2006). Therefore, the increased mRNA expression induced by altered DNA methylation of *IL6* as a result of n-3 PUFA supplementation and exercise may result in an anti-inflammatory response; however, these results need to be supported by future work examining the impact of exercise and n-3 PUFAs on the regulation of pro-inflammatory cytokines.

5.5 Conclusion

Despite reduced mRNA expression of the DMNT enzymes following exercise, there was no impact of exercise on global or gene-specific DNA methylation. Potentially a measure of DNMT enzyme activity may explain the lack of agreement between mRNA expression and DNA methylation. The supplementation of n-3 PUFAs altered the composition of FAs in whole blood and significantly correlated with *IL6* DNA methylation; however, the absence of inflammatory cytokine protein concentrations prevents detecting the impact of supplementation on inflammation. A relationship between exercise and supplementation was detected for *IL6* DNA methylation and mRNA expression suggesting a potential anti-inflammatory role for exercise-induced expression. Further work is needed to confirm the results in this chapter. Although mRNA expression has been used to determine the impact of modified DNA methylation, the inclusion of phenotypes related to exercise performance and inflammation would allow the functional significance of changes in DNA methylation to be determined.

Chapter 6 Impact of aerobic exercise and fatty acid supplementation on global and gene-specific DNA methylation.

The work contained in this chapter has previously been published (Appendix A):

David John Hunter, Lynsey James, Bethan Hussey, Alex J. Wadley, Martin R. Lindley & Sarabjit S. Mastana (2019) Impact of aerobic exercise and fatty acid supplementation on global and gene-specific DNA methylation, Epigenetics, DOI: 10.1080/15592294.2019.1582276

6.1 Introduction

In chapter 5, a lack of association between an acute bout of exercise to volitional fatigue and both global and gene-specific (*PPARGC1A* and *IL6*) DNA methylation was detected. Whereas following the supplementation of n-3 PUFAs, exercise was sufficient to reduce the DNA methylation of a single CpG site of *IL6* and increase the mRNA expression. Seminal work by Barres et al., (2012) determined that the bout of exercise to volitional fatigue was sufficient to alter global methylation patterns, whereas, a candidate gene approach determined a high but not low intensity bout of exercise altered DNA methylation of genes associated with mitochondrial biogenesis. In this chapter, we sought to determine whether the lack of modulated DNA methylation in the previous chapter was the result of an insufficient exercise stimulus.

Environmental stimuli, including exercise and dietary interventions, can modify the DNA methylome at a global and gene-specific level (Alegría-Torres et al. 2011). Exercise training studies have demonstrated hypomethylation of the genome following exercise in both skeletal muscle (Nitert et al. 2012; Rowlands et al. 2014; Seaborne et al. 2018) and blood leukocytes (Denham et al. 2016; Denham, O'Brien, Marques, et al. 2015; Dimauro et al. 2016). Within skeletal muscle, acute exercise has been demonstrated to induce hypomethylation (Bajpeyi et al. 2017; Barrès et al. 2012; Seaborne et al. 2018); however, the only investigation of DNA methylation in leukocytes following acute exercise failed to detect any changes in DNA methylation (Robson-Ansley et al. 2014). Despite the scarcity of literature surrounding the impact of acute exercise on DNA methylation in leukocytes, an epigenetic consequence is suggested by the remodelling of the leukocyte transcriptome following acute exercise (Büttner et al. 2007; Connolly et al. 2004; Gjevestad et al. 2015).

Acute exercise is associated with adjustments in the expression of genes involved in a variety of cellular processes, including immune response, mitochondrial biogenesis, metabolism and muscle remodelling (Booth, Chakravarthy, and Spangenburg 2002; Egan and Zierath 2013; Gjevestad et al. 2015). The *PPARGC1A* gene, which encodes for PGC-1α, is known as the master regulator of mitochondrial biogenesis and plays an important role in aerobic training adaptation (Ventura-Clapier et al. 2008). In immune cells, *PPARGC1A* is associated with anti-inflammatory (Thomas et al. 2012;

Yakeu et al. 2010) and anti-oxidant defence (Ferrer et al. 2009); however, the impact of exercise-induced inflammation and oxidative stress on *PPARGC1A* DNA methylation is unknown. Epigenetic studies have linked a CpG site -260 bases from the promoter of *PPARCG1A* with the regulation of mRNA expression. In skeletal muscle, exercise can demethylate the *PPARGC1A* -260 CpG site which has been shown to concurrently upregulate *PPARGC1A* mRNA expression (Bajpeyi et al. 2017; Barrès et al. 2012). Although well characterised in skeletal muscle, the regulation of *PPARGC1A* expression in other cells and tissues, including immune cells is poorly understood (Busquets-Cortés et al. 2017).

Exercise of sufficient intensity and duration can cause tissue injury and lead to a systemic inflammatory response (Gjevestad et al. 2015; Gleeson et al. 2011). Increased circulating levels of the inflammatory cytokines IL-6 and TNF-α are strongly correlated with the progression of sarcopenia and measures of physical performance (Cesari et al. 2004; Visser et al. 2002). Acute exercise can also increase the production of reactive oxygen species, in both skeletal muscle and immune cells (Powers, Nelson, and Hudson 2011), potentially leading to the development of oxidative stress and damage to lipids, proteins and DNA (He et al. 2016). Increases in markers of oxidative stress and circulating levels of inflammatory cytokines, such as IL-6 and TNF-α , have been shown to alter the expression of DNMTs (Angelini et al. 2017; Braconi, Huang, and Patel 2010; Foran et al. 2010; Hodge et al. 2001; Horsburgh et al. 2015) and influence DNA methylation patterns (Robson-Ansley et al. 2014; Sharples et al. 2016). DNA methylation of inflammatory cytokines have been associated with various inflammatory diseases including *IL6* with Rheumatoid Arthritis (Nile et al. 2008) and obesity (Na et al. 2015); *TNF* DNA methylation with type 2 diabetes (Zhang et al. 2017) and Alzheimer's disease (Kaut et al. 2014). Despite increased circulating levels of inflammatory cytokines post-exercise (Gjevestad et al. 2015; Gleeson et al. 2011), the impact of exercise on the DNA methylation of genes encoding inflammatory cytokines such as *IL6* and *TNF* remains unknown.

There is the potential for the dietary supplementation of FAs to prevent exercise-induced inflammation via the modulation of DNA methylation. Supplementation of FAs, including n-3 PUFAs and extra virgin olive oil (EVOO), are consumed to reduce levels of inflammation (Calder 2015; Rosignoli et al. 2013); however, the impact of these

supplements on exercise-induced inflammation is equivocal. Some studies have detected reductions in inflammation post-exercise with FA supplementation (Marques et al. 2015; Mickleborough et al. 2015), whereas, others have reported no change in inflammation (Martorell et al. 2014; Nieman et al. 2009). An emerging mechanism for the anti-inflammatory impact of FA supplementation is via epigenetic modifications (Ma et al. 2016; Saini et al. 2017; Tartibian et al. 2011; Tremblay et al. 2017). The supplementation of the diet with krill oil, high in n-3 PUFAs, has been demonstrated to reduce *PPARGC1A* mRNA expression and the change in mRNA expression was negatively correlated to the change in plasma n-3 PUFAs (Rundblad et al. 2018). Total n-3 PUFA content is negatively correlated to both *IL6* DNA methylation and IL-6 protein concentration (Ma et al. 2016). EVOO is a commonly used control in exercise studies to assess the impact of n-3 PUFA; however, the supplementation of EVOO has also been reported to modify the DNA methylation of genes associated with inflammation (Arpón et al. 2017). It remains to be identified whether the supplementation of FAs have an epigenetic impact on exercise-induced inflammation.

Aim

The primary aim of this chapter is to investigate the impact of acute exercise and FA supplementation on leukocyte DNA methylation and mRNA expression. We also investigated whether these relationships impacted physiological variables related to exercise performance, inflammation and oxidative stress.

Objectives

1. Assess whether an acute bout of exercise and FA is sufficient to alter global DNA methylation and mRNA expression of DNMT enzymes.
2. Determine any changes in gene-specific methylation (*PPARGC1A*, *IL6* and *TNF*) and subsequently mRNA expression as a result of exercise and FA supplementation.
3. Identify any association between physiological variables and modulated DNA methylation.

6.2 Methods

The experimental procedures for this study were approved by the Loughborough University Ethics Human Participants sub-committee (Study ID: R14-P72).

6.2.1 Participants

Ten healthy male trained cyclists were recruited into the study according to section 3.1. Complete data set were available for eight participants whose characteristics are described in Table 6.1.

Table 6.1 - *Participant characteristics. $\dot{V}O_{2max}$, maximum aerobic uptake; Wmax, maximal aerobic power*

Variable	Mean ± SD (n = 8)
Age (yrs)	39.50 ± 5.90
Body Mass (kg)	73.04 ± 8.31
Height (m)	1.74 ± 0.84
BMI ($kg \cdot m^{-2}$)	24.07 ± 2.46
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	53.88 ± 5.24
Wmax (W)	321.63 ± 28.15

6.2.2 Study overview

The study consisted of a familiarisation session and four experimental trials. Experimental trials were completed before and after a four-week supplementation of n-3 PUFA and EVOO (Before n-3 PUFA, After n-3PUFA, Before EVOO and After EVOO) in a double-blind, randomised, repeated measures design. A four-week washout was included between each supplementation period (Figure 6.1A).

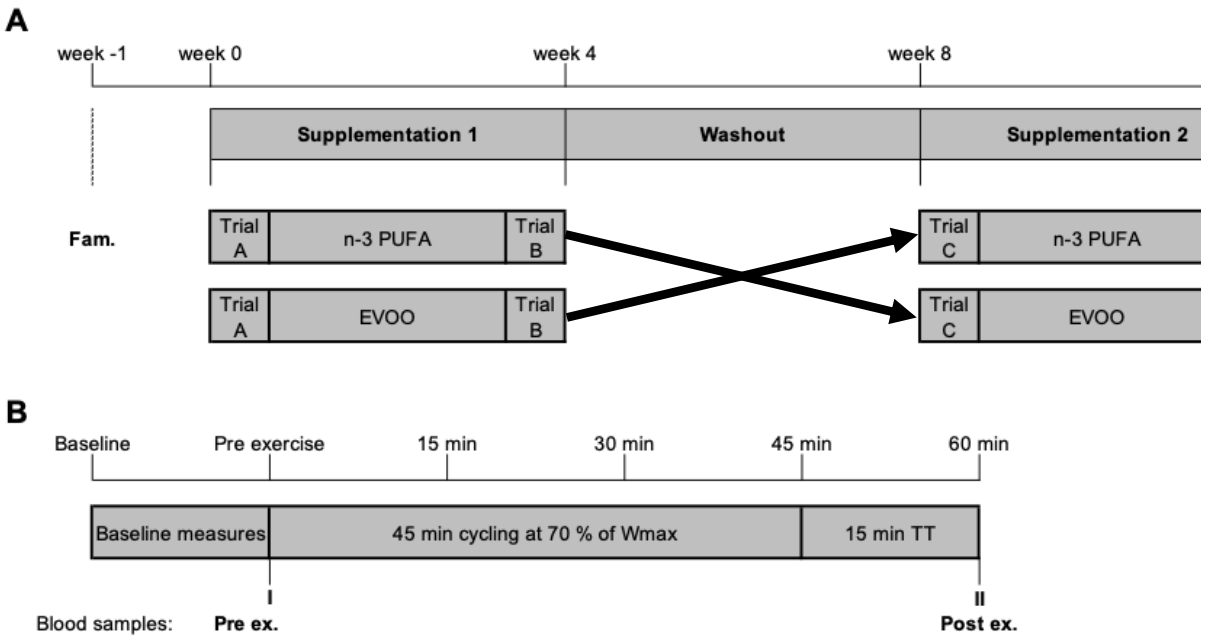

Figure 6.1 – *Schematic representation of (A) the study outline and (B) trial day. n-3 PUFA, omega-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil; Wmax, maximal aerobic power; TT, time trial.*

6.2.2.1 Familiarisation

Participants underwent anthropometric assessment for height, body mass (section 3.2.1) and eight-skinfold measurements according to the International Society for the Advancement of Kinanthropometry (ISAK) prior to the start of the study. Maximal aerobic power (Wmax) and maximal oxygen uptake ($\dot{V}O_{2max}$) were determined using a graded exercise test on a Lode Excalibur Sport ergometer (Lode B.V, Netherlands). The exercise test began with a warm-up period of 5-min cycling at 100 W. Workload then increased by 50 W every 3-min until volitional fatigue (decrease in the self-selected cadence of 20 revs·min^{-1}). Expired air was collected in the final minute of each stage to allow $\dot{V}O_{2max}$ determination using primary and secondary criteria (Howley, Bassett, and Welch 1995). Wmax was calculated using the formula:

$$Wmax = Workload \div [(t/180) \times 50]$$

Where t is the time in seconds completed in the final stage. Following the completion of the incremental cycling test, participants received a 10-minute rest before completing a 15-minute time-trial (TT) familiarisation.

6.2.2.2 Experimental trials

Pre-trial standardisation of diet and exercise was performed according to section 3.2.2. Figure 6.1B provides a schematic representation of the trial day. On arrival to the laboratory, baseline measures were recorded (section 3.2.1) and an intravenous catheter was inserted for the collection of blood samples (section 3.3). Following the collection of a baseline blood sample (Pre-ex), participants completed the exercise bout consisting of 45-minutes cycling at 70% Wmax, followed by a 15-minute TT (Jeukendrup et al. 1996). The electromagnetically braked ergometer was set in hyperbolic mode for the initial 45-minutes cycling at 70% Wmax to ensure that work rate was constant and independent of cadence. Upon completion of the 45-minutes of steady state cycling, the ergometer was changed linear mode for the 15-minute TT, where the work rate is dependent on the pedalling rate, allowing the participants to maximise power output by maintaining a high cadence. The linear factor was selected using the following formula:

$$W = L \times (RPM)^2$$

Where RPM is the pedalling rate from the $\dot{V}O_{2max}$ test. Verbal encouragement and feedback on elapsed time were provided throughout the TT; however, no feedback on power output, heart rate or cadence were provided. The total work done and mean power throughout the 15-min TT were calculated and used a measure of performance. Upon completion of the exercise bout a further blood sample was collected (Post-ex).

6.2.2.3 Supplementation

Both n-3 PUFA (Holland and Barrett, UK) and EVOO (Puritan's Pride, USA) supplements were provided in capsule form. Participants were instructed to take six capsules per day providing 5.7g of n-3 PUFA (4.08g of EPA and 1.62g of DHA) and 0.01g per day of α-Tocopherol or 6 g per day of EVOO. The n-3 PUFA dose was chosen based on previous findings showing the dose was sufficient to induce changes in the FA profile of human blood (McGlory et al. 2014; Metherel et al. 2009). Returned capsules were counted to determine the compliance of supplementation.

6.2.3 Analytical Procedures

6.2.3.1 Blood sampling

Venous blood samples were collected (section 3.3) for the assessment of DNA methylation, mRNA expression and both IL-6 and carbonyl protein concentrations via an intravenous catheter (section 3.3) at Pre-ex and Post-ex in each of the four trials (Before n-3PUFA, After n-3PUFA, Before EVOO and After EVOO).

6.2.3.2 DNA methylation

Genomic DNA was extracted from whole blood (section 3.4.1). The concentration of isolated gDNA was 183.50 (± 54.48) ng/uL and an A_{260}/A_{280} ratio of 1.90 (± 0.02). Global DNA methylation was assessed using LUMA (section 3.4.2). For determination of gene-specific DNA methylation, DNA was bisulfite-converted (section 3.4.1) and underwent PCR using the PyroMark PCR kit. DNA methylation percentage was determined via pyrosequencing (section 3.4.3). The assays used to determine DNA methylation are presented in Table 4.2 (*PPARGC1A, IL6 and TNF*).

6.2.3.3 Analysis of mRNA expression

RNA was extracted from whole blood. The concentration of isolated RNA was 120.32 (± 41.02) ng/uL and an A_{260}/A_{280} ratio of 2.09 (± 0.02). RNA was then cDNA converted (section 3.4.5), and relative mRNA expression was performed using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001) using GAPDH as the reference gene (section 3.4.6). Primer sequences for the assays used to determine mRNA expression of the genes of interest are displayed in Table 4.3 (*GAPDH, Total PPARGC1A, IL6, TNF, DNMT1, DNMT3a and DNMT3b*). The mean Ct value of GAPDH across all participants and experimental conditions was 17.134 (±0.41) with a low variation of 2.40%.

6.2.3.4 IL-6

Serum IL-6 concentration was determined Pre-ex and Post-ex for each of the four trials using the high sensitivity enzyme immunoassay kits (R & D Systems, USA). Haematocrit and haemoglobin values were used to ascertain plasma volume changes that were used to adjust serum IL-6 concentrations (Dill and Costill 1974).

6.2.3.5 Protein Carbonyls (PC)

PBMCs, isolated from whole blood by density gradient centrifugation using Ficoll-Paque Premium (GE healthcare, USA) according to the manufacturer's instructions, and serum (section 3.3) were assessed by an in-house ELISA (Buss et al. 1997; Carty et al. 2000). Serum samples, PBMC lysates and standards were diluted in coating buffer (50mM sodium carbonate, pH = 9.2) to a concentration of 0.05mg/mL using the bicinchoninic assay method. Protein carbonyls groups were derivatised with 2, 4-dinitrophenylhydrazine (1mM, in 2M HCl) and incubated with monoclonal mouse anti-DNP antibody (Sigma Aldrich, UK) and rat anti-mouse IgE, conjugated to HRP (AbD Serotec, UK). Well absorbance was measured at 490nm and the PC concentration determined by using absorbance values of known PC standards made in our laboratory (1.28-5.20 nmol/mg protein). PC concentration in PBMCs were adjusted for changes in protein concentration and cell number (Beckman Coulter, UK) induced by acute exercise.

6.2.4 Statistical Analysis

Statistical analysis was performed according to section 3.5. DNA methylation, mRNA expression values and physiological variables related to exercise performance, inflammation and oxidative stress were analysed using a 2 (supplement) x 2 (trial) x 2 (time) RM-ANOVA. The impact of exercise is presented using the absolute values (mean of all trials for each time point), whereas, the impact of supplementation of FAs is presented as the relative change (Δ) between before and after supplementation trials (after supplementation – before supplementation). Values represented as mean $\pm$ 95% CI.

Spearman's Rho correlation analysis was used to assess the relationship between DNA methylation and mRNA expression and physiological markers related to exercise performance, inflammation and oxidative stress. Moderate (>0.5) correlation coefficients were considered to be of interest; however, only large (> 0.7) correlation coefficients were deemed statistically significant. All data presented as mean ± 95% CI unless otherwise stated.

6.3 Results

6.3.1 Physiological responses - Exercise performance, inflammation and oxidative stress

Supplementation of FAs did not alter the work done (Δ n-3PUFA = 7.9 kJ, Δ EVOO = -9.6 kJ; p = 0.06) or mean power (Δ n-3PUFA = -0.25W, Δ EVOO = -10.96 W; p = 0.101) during the TT. There was a significant increase in serum IL-6 in response to exercise (Pre-ex: 0.63 ± 0.24 pg/mL, Post-ex: 3.78 ± 0.55 pg/mL; p < 0.001); however, the supplementation of FAs had no impact on *IL6* protein concentrations (p > 0.05). Decreased PBMC PC was detected following exercise (Pre-ex: 2.15 ± 0.20, Post-ex: 1.26 ± 0.17; p < 0.001), whereas, exercise had no impact on the serum PC concentration (p > 0.05). Supplementation of FAs had no impact on serum or PBMC PC concentrations (p > 0.05).

6.3.2 Global cytosine methylation and DNMT mRNA expression

One-hour of cycling reduced global methylation, assessed by LUMA (Figure 6.2A; Pre-ex: 79.2%; Post-ex: 78.7%, p = 0.008), and the mRNA expression of both *DNMT3a* (Figure 6.2C; p = 0.018) and *DNMT3b* (Figure 6.2D; p = 0.046). Supplementation of FAs did not alter global methylation (Before n-3PUFA: 79.05%, After n-3PUFA: 78.82%, Before EVOO: 79.12%, After EVOO: 79.11%; Figure 6.3A; p > 0.05) or mRNA expression of *DNMT3a* or *DNMT3b* (Figure 6.3; p > 0.05). While *DNMT1* mRNA expression was unaffected by exercise, a significant interaction was identified between supplement and trial (p = 0.048; Figure 6.3B) indicating differential effects on mRNA expression with the two supplements. No correlation was detected between global DNA methylation values and *DNMT* mRNA expression.

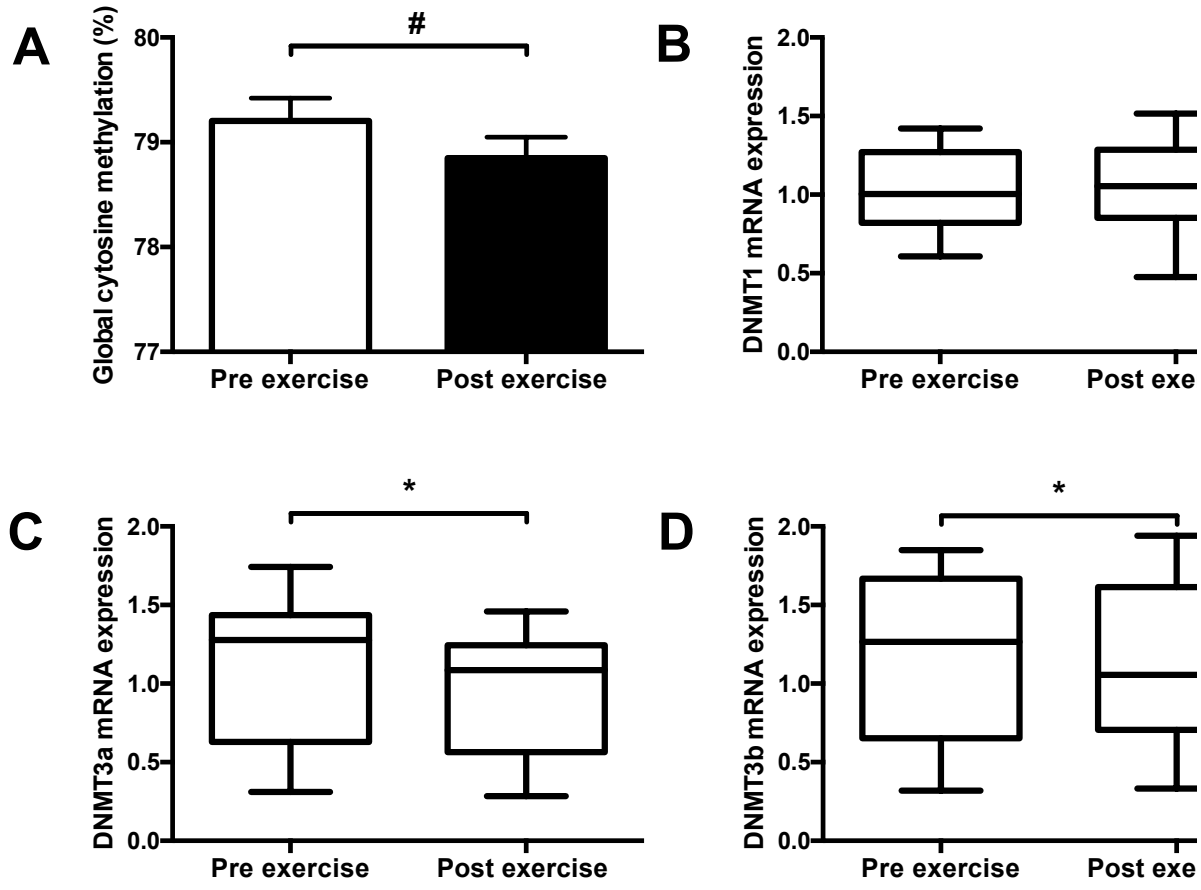

Figure 6.2 – *Effect of exercise on (A) global DNA methylation and mRNA expression of (B) DNMT1, (C) DNMT3a and (D) DNMT3b. Data presented as the mean value of all trials for each time point. * p <0.05, # p <0.01*

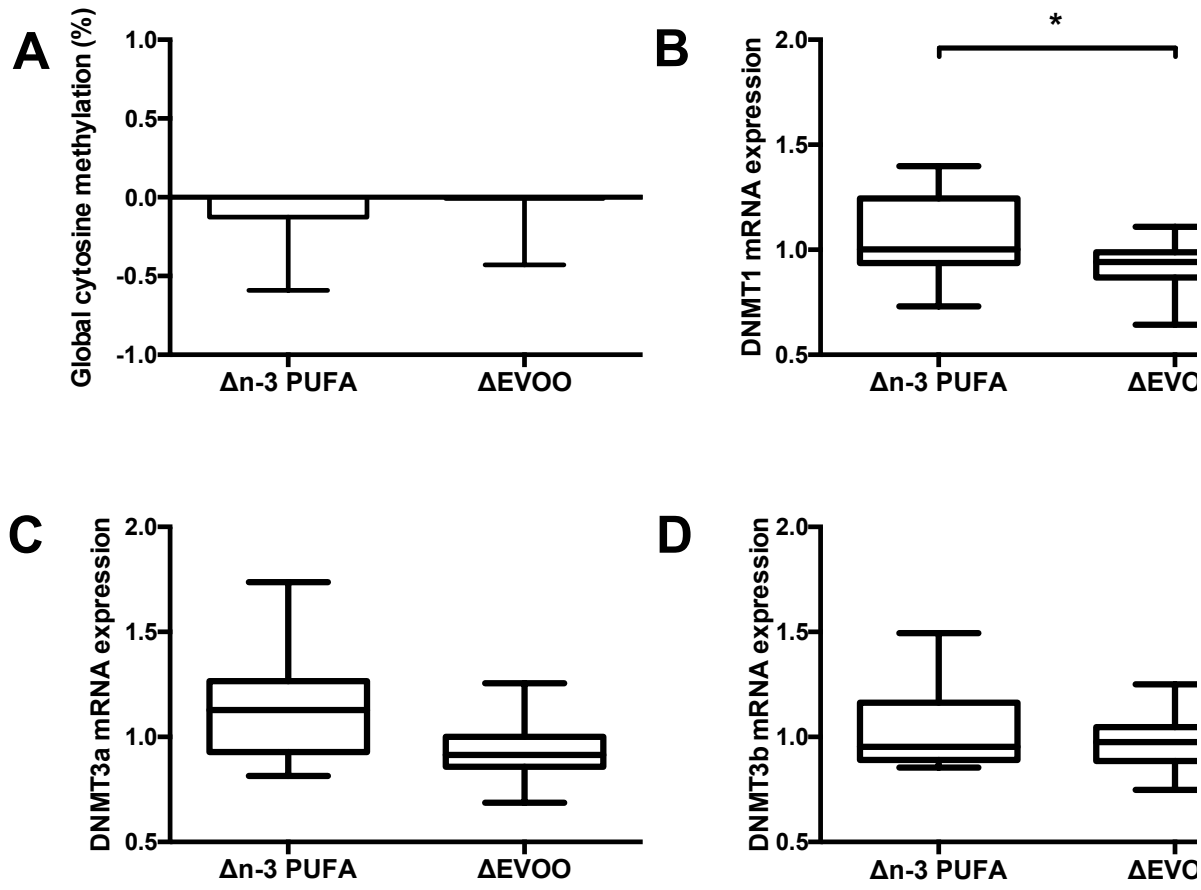

Figure 6.3 - *The impact of supplementation of n-3 PUFA and EVOO on (A) global DNA methylation and mRNA expression of (B)DNMT1, (C)DNMT3a and (D)DNMT3b. Data presented as the relative change (Δ) between before and after supplementation trials for each supplement. n-3 PUFA, n-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil. * p <0.05.*

6.3.3 Gene-specific DNA Methylation and mRNA expression

6.3.3.1 PPARGC1A

A reduction in *PPARGC1A* DNA methylation (Pre-ex: 6.9%; Post-ex: 6.3%, Figure 6.4A; $p < 0.001$) and an increase in mRNA expression (Figure 6.4B; $p < 0.001$) were detected following exercise. The supplementation of FAs had no impact on *PPARGC1A* DNA methylation or mRNA expression ($p > 0.05$). Moderate but non-significant negative correlations were detected between *PPARGC1A* DNA methylation and *DNMT3a* and *DNMT3b* mRNA expression (Figure 6.6).

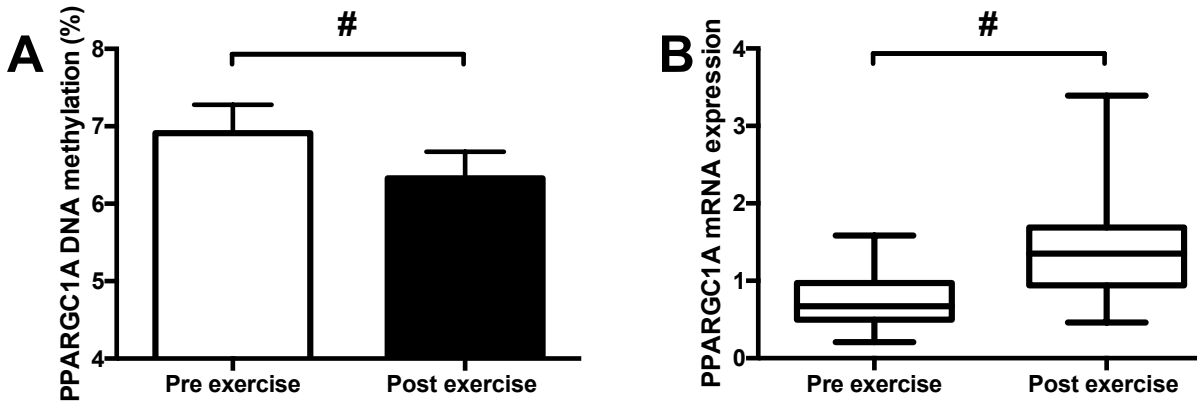

Figure 6.4 - *Effect of exercise on (A) DNA methylation of CpG-260 and (B) mRNA expression of PPARGC1A. Data presented as the mean value of all trials for each time point. # p < 0.01*

6.3.3.2 IL6

Despite an increase in IL-6 protein concentrations following exercise, there was no change in *IL6* DNA methylation (p > 0.05) or mRNA expression (p > 0.05) following exercise. A significant interaction was detected between supplement and trial for CpG3 (-1094) indicating increased DNA methylation following n-3 PUFA and decreased methylation following EVOO (Before n-3PUFA: 92.90%, After n-3PUFA: 93.32%, Before EVOO: 93.49%, After EVOO: 93.02%; Figure 6.5A; p = 0.038). A similar, non-significant (p = 0.080) trend was detected for *IL6* mRNA expression following supplementation (Figure 6.5B). A significant correlation was detected between the mean *IL6* methylation across all CpG sites and *DNMT3b* mRNA expression (Figure 6.6, p = 0.007).

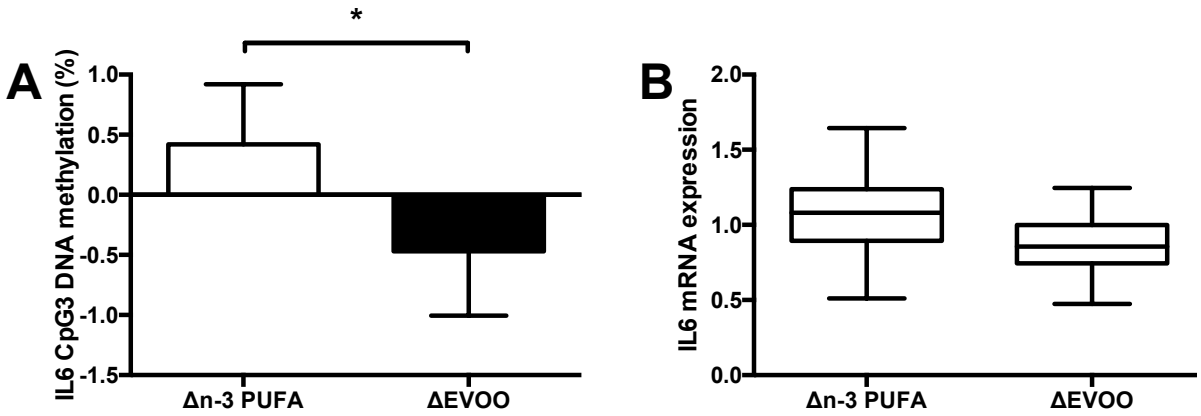

Figure 6.5 - *The impact of n-3 PUFA and EVOO supplementation on (A) IL6 CpG3 DNA methylation and (B) IL6 mRNA expression. Data presented as the change (Δ) between before and after supplementation trials. n-3 PUFA, n-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil. * p <0.05.*

6.3.3.3 TNF

Neither exercise or the supplementation of FAs altered *TNF* DNA methylation or mRNA expression. Trends were identified between 3 *TNF* CpG sites and differential methylation following supplementation (CpG2 p = 0.069; CpG3 p = 0.098; CpG4 p = 0.067; CpGmean p = 0.077). *TNF* DNA methylation was negatively correlated with *TNF* mRNA expression (Figure 6.6; p = 0.007). Moderate, however, non-significant correlations were detected between both *IL6* and *DNMT3a* mRNA expression, and *TNF* DNA methylation (Figure 6.6).

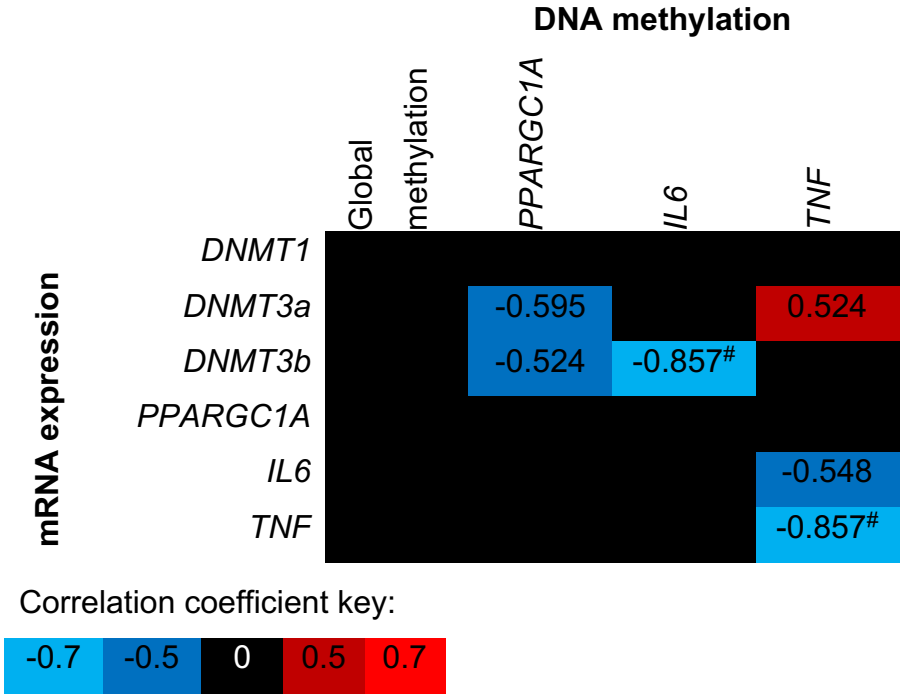

Figure 6.6 – *Spearman's Rho correlation coefficients between mean DNA methylation values and gene expression values across all conditions (supplement, time and trial). The mean of all CpG sites assessed for each gene has been used to provide an overall view of the region of interest. Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. * p < 0.05, # p < 0.01*

6.3.4 Associations between DNA methylation and post-exercise physiological markers

Figure 6.7 demonstrates the association between post-exercise DNA methylation and physiological markers related to exercise, oxidative stress and inflammation. Prior to FA supplementation, both *PPARGC1A* and *TNF* methylation post-exercise are significantly correlated with Time Trial (TT) performance (Figure 6.7, $p < 0.05$). Following the supplementation of n-3 PUFA and EVOO, correlations between TT performance and both *PPARGC1A* and *TNF* DNA methylation are weakened and no longer significant (Figure 6.7). A negative correlation was detected between PBMC PC concentration, an intracellular measure of oxidative stress, and both global and *PPARGC1A* methylation prior to supplementation of FAs; however, no association was detected following n-3 PUFA supplementation (Figure 6.7). The concentration of PC in serum, a systemic measure of oxidative stress, was uncorrelated with DNA methylation at baseline; however, following EVOO supplementation significant correlations existed between serum PCs and both *PPARGC1A* and *TNF* DNA methylation (Figure 6.7). The only significant correlation between DNA methylation and serum IL-6 concentration was a negative correlation with global DNA methylation following n-3 PUFA supplementation (Figure 6.7).

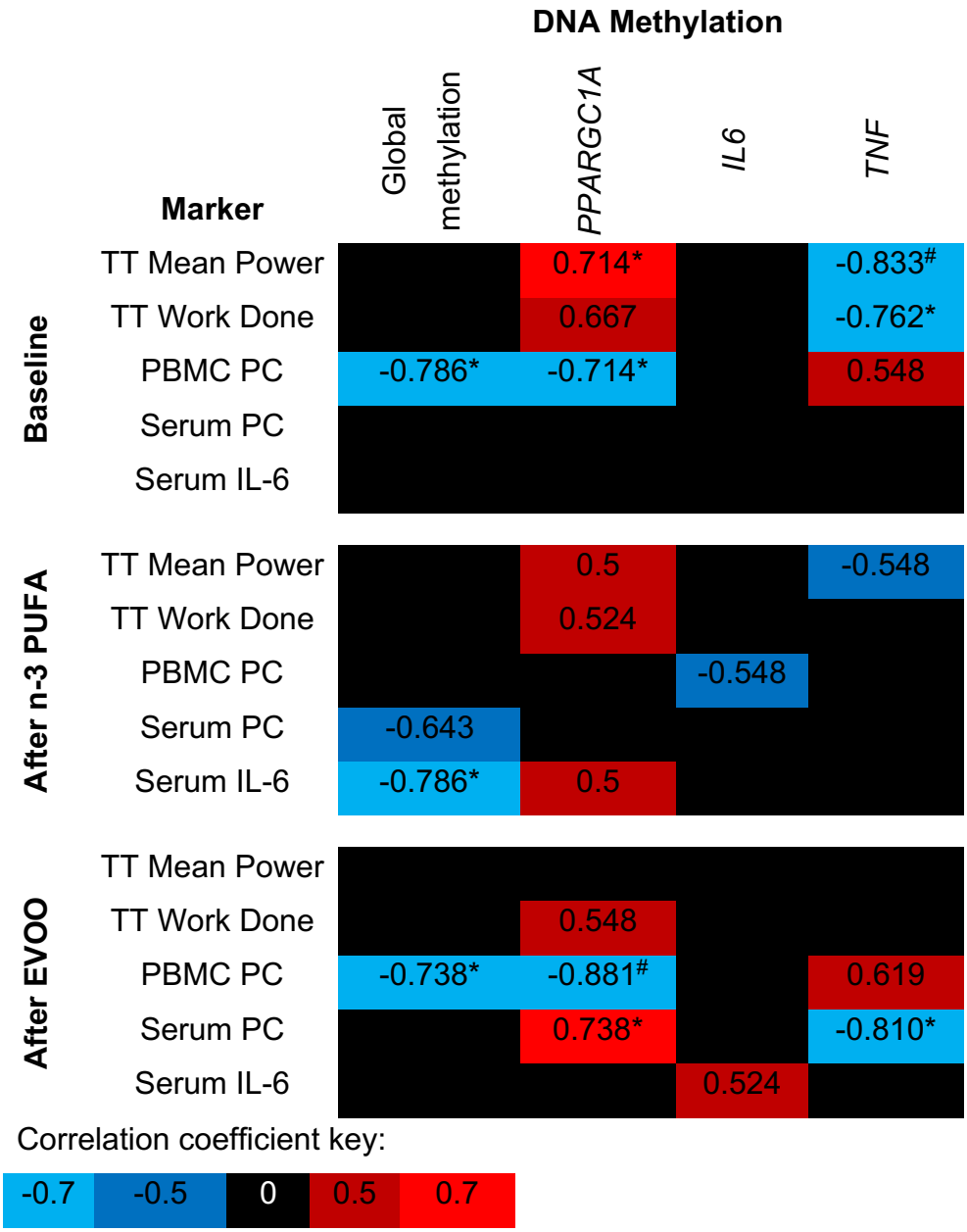

Figure 6.7 – *Spearman's Rho between post-exercise DNA methylation and physiological markers related to exercise performance, oxidative stress and inflammation. The mean of all CpG sites assessed for each gene has been used to provide an overall view of the region of interest. Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. n-3 PUFA, omega-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil; TT, Time trial; PC, protein carbonyl. *p < 0.05, # p < 0.01.*

6.4 Discussion

A single bout of aerobic exercise and supplementation of FAs can modulate leukocyte DNA methylation and mRNA expression patterns. A one-hour cycling bout decreased global and *PPARGC1A* DNA methylation and mRNA expression of *DNMT3a*, *DNMT3b* and *PPARGC1A*. The supplementation of FAs induced differential effects on the DNA methylation of a CpG site in the promoter region of *IL6*; n-3 PUFA increased methylation, whereas, EVOO supplementation decreased methylation. The same result was identified for mRNA expression of *DNMT1* and trends existed for 3 CpG sites in the promoter region *TNF* . Significant correlations were identified between global DNA methylation; *PPARGC1A*, *IL6* and *TNF* DNA methylation post-exercise; and physiological markers related to exercise performance, inflammation and oxidative stress indicating that the epigenetic modifications have functional effects.

For the first time we report, global hypomethylation in leukocytes following an acute bout of exercise. The only previous study to investigate the impact of acute exercise in blood cells failed to detect any change in DNA methylation following correction for multiple testing (Robson-Ansley et al. 2014). Whereas, the results of the present study are in accordance with previous reports of a net hypomethylation following acute bouts of exercise in plasma (da Silva et al. 2017), skeletal muscle (Barrès et al. 2012; Seaborne et al. 2018) and chronic exercise training studies (Denham et al. 2016; Denham, O'Brien, Marques, et al. 2015; Dimauro et al. 2016; Nitert et al. 2012; Rowlands et al. 2014; Seaborne et al. 2018). Other studies have failed to detect any change in global DNA methylation (King-Himmelreich et al. 2016; Lindholm et al. 2015); however, this can be explained by a similar number of CpG sites increasing and decreasing in DNA methylation (Lindholm et al. 2015). It has also been demonstrated that exercise-induced hypomethylation is retained during periods of detraining, allowing it to become further hypomethylated following further training (Seaborne et al. 2018). These data suggest that both acute and chronic exercise is sufficient to alter DNA methylation patterns typically resulting in hypomethylation.

In the present study, a 4-week supplementation of FAs did not influence global DNA methylation. In contrast, a 6-month supplementation of n-3 PUFA decreased *LINE-1* DNA methylation, a surrogate for global DNA methylation, in Alzheimer's patients

(Karimi et al. 2017). However, *LINE-1* methylation is increased in Alzheimer's patients compared to healthy controls (Di Francesco et al. 2015); therefore, the supplementation of n-3 PUFA in these individuals may act to restore global DNA methylation to the normal level detected in healthy individuals. The use of different surrogate measures of global methylation (LUMA vs *LINE-1*) prevents the direct comparison between studies because of the different region which these assays investigate. Two separate studies have indicated that the methylation estimates provided by LINE-1 and LUMA are poorly correlated (Lisanti et al. 2013; Wu et al. 2011).

For the first time, post-exercise decreased methylation and concurrent increased mRNA expression of *PPARGC1A* following a bout of aerobic exercise have been detected in leukocytes. The results from the present study match previous reports of aerobic exercise-induced hypomethylation in skeletal muscle (Bajpeyi et al. 2017; Barrès et al. 2012; Nitert et al. 2012) potentially indicating a systemic impact of exercise on *PPARGC1A* DNA methylation. The mRNA expression profile of skeletal muscle and PBMCs are highly associated following an 8-week supplementation of n-3 PUFAs (Rudkowska et al. 2011). Although we do not find any association with *PPARGC1A* methylation / mRNA expression and n-3 PUFA supplementation in the present study, the hypomethylation detected in the present study is consistent with the impact of exercise in skeletal muscle providing further evidence for blood-derived expression profiles to be used as a surrogate for skeletal muscle.

The only previous report of *PPARGC1A* methylation from leukocytes failed to detect an association with physical activity (Clarke-Harris et al. 2014). The lack of previous association could be the result of the investigation of different CpG sites in the promoter region of *PPARGC1A*. Alternatively, the discordance in these results could reflect the heterogeneity in the methylation pattern of immune cells (Jones et al. 2017). Exercise increases the number of circulating leukocytes; therefore, changes in methylation may be the result of different proportions of leukocytes rather than a change in DNA methylation patterns (Jaffe and Irizarry 2014). The present study has adjusted DNA methylation values to account for the number of leukocytes (lymphocytes, neutrophils, monocytes, basophils and eosinophils) (Jones et al. 2017), whereas, previous reports have failed to account for this critical variable.

The positive correlation between leukocyte *PPARGC1A* methylation and exercise performance indicates that increased DNA methylation may provide a performance advantage. *PPARGC1A* is thought to upregulate mitochondrial biogenesis in monocytes to induce a shift towards an anti-inflammatory phenotype (Thomas et al. 2012; Yakeu et al. 2010) and antioxidant defence in lymphocytes (Ferrer et al. 2009). Although we did not find an association with IL-6 protein concentration, a negative association was detected between *PPARGC1A* DNA methylation and PC concentration indicating potential epigenetic control of the antioxidant role of *PPARGC1A*. There is limited literature comparing mitochondrial function in leukocytes and skeletal muscle following exercise; however, the association between gait speed and mitochondrial function in both skeletal muscle tissue and PBMCs provides a conserved mechanism between mitochondrial function in skeletal muscle and blood-derived mitochondria (Tyrrell et al. 2015). Further evidence of a conserved mechanism is suggested with genes related to mitochondrial structure and function found to be co-expressed in skeletal muscle and neutrophils following aerobic exercise (Broadbent et al. 2017). Future studies are required to detect if the same phenotypic associations exist in skeletal muscle as detected in leukocytes in the present study.

Aerobic exercise did not alter the DNA methylation or mRNA expression of either *IL6* or *TNF*. The epigenetic impact of exercise on inflammatory cytokines is relatively unknown; however, several studies have indicated a role for cytokine DNA methylation in inflammatory disease (Kaut et al. 2014; Na et al. 2015; Nile et al. 2008; Zhang et al. 2017). Although no association between *TNF* DNA methylation and mRNA expression was detected in the present study, n-3 PUFAs have previously been demonstrated to reverse the epigenetic changes observed with inflammation in skeletal muscle cells. The administration of *TNF* induced hypermethylation and decreased mRNA expression of MyoD (Sharples et al. 2016), whereas the supplementation of EPA dampens the impact of *TNF* in muscle and restores MyoD mRNA expression (Saini et al. 2017). Despite an increase in the circulating protein concentration of IL-6 in the present study, the exercise bout may not increase TNF-α protein concentration and subsequently induce an inflammatory response sufficient to modify DNA methylation patterns of inflammatory cytokines. *TNF* hypermethylation is reported in elderly individuals who maintained or increased their energy expenditure by 500 kcal/wk over

an 8-year period (Shaw et al. 2014). The same *TNF* CpG sites as the present study have previously been shown to negatively associate with mRNA expression, plasma concentrations and measures of adiposity (Hermsdorff et al. 2013; Marques-Rocha et al. 2016). In the present study, a significant negative correlation was detected between *TNF* DNA methylation post-exercise and BMI, exercise performance and *TNF* mRNA expression. These data suggest an acute bout of exercise may not regulate *TNF* DNA methylation; however, the long-term benefits of regular exercise, such as reduced adiposity, may subsequently increase *TNF* DNA methylation levels and as a result, reduce *TNF* mRNA expression and the chronic low-grade inflammation levels associated with increased adiposity.

Previously decreased methylation in a region ~600 bp upstream of the *IL6* promoter has been associated with increased erythrocyte n-3 PUFA concentrations and mRNA expression (Ma et al. 2016). In the present study, the supplementation of EVOO and n-3 PUFA had contrasting effects on a single CpG (-1094) of *IL6* (increased methylation following n-3 PUFA and decreased methylation with EVOO). The region ~1,000 bp from upstream of was investigated in the present study because of previous associations between DNA methylation and both inflammatory diseases (Na et al. 2015; Nile et al. 2008) and mRNA expression (Nile et al. 2008). Conflicting results between studies may indicate that distinct regions of the promoter regulate *IL6* expression differently. Supplementation of n-3 PUFA and OO have been shown to induce differential methylation of *ELOVL* and *FADS* genes which are responsible for the metabolism of FAs (Hoile *et al.*, 2014). The differential DNA methylation of these enzymes indicates the potential for n-3 PUFAs to switch towards the production of less inflammatory eicosanoids. Although the DNA methylation of *FADS* and *ELOVL* genes have not been measured in the present study, a switch towards n-3 PUFA derived eicosanoid production, such as 3-series rather than 2-series prostaglandins, has been shown to reduce cytokine expression (Calder 2015) which is potentially indicated by the increased DNA methylation of *IL6* following n-3 PUFA, but not EVOO, supplementation.

The impact of exercise and FA supplementation on *DNMT* mRNA expression was investigated to identify whether changes in *DNMT* mRNA expression could be a potential mechanism underlying modulated DNA methylation. *DNMT1* mRNA

expression was modulated by FA supplementation, whereas, exercise reduced the expression of both *DNMT3a* and *DNMT3b*. This is the first demonstration of reduced expression of *DNMT3a* following acute exercise, whereas, the reduction in *DNMT3b* expression has previously been reported (Horsburgh et al. 2015; Laye and Pedersen 2010). The inclusion of DNA methylation assessment in the present study allows the confirmation that following a single bout of aerobic exercise *DNMT* expression is decreased alongside decreases in global and gene-specific DNA methylation. The only previous report of a concurrent assessment of exercise-induced *DNMT* expression and DNA methylation was following an 8-week resistance training program (Denham et al. 2016). The genome-wide method of methylation does not identify a net increase or decrease in global methylation; therefore, further studies are required to identify whether the modulation of *DNMT3b* causes hypomethylation or if it is essential in both hyper- and hypomethylation.

The present study detects opposing effects of n-3 PUFA and EVOO supplementation on *DNMT1* mRNA expression. There is a paucity of literature surrounding the impact the FA supplementation and *DNMT* expression in humans; whereas, animal models have associated supplementation of alpha-linolenic acid, an n-3 PUFA, with changes in *DNMT* mRNA expression (Niculescu, Lupu, and Craciunescu 2013, 2014). Interestingly, similar to the present study, no change in global DNA methylation was detected alongside modulated *DNMT1* expression (Niculescu et al. 2014). A change in global DNA methylation potentially would not be expected with increased in *DNMT1* mRNA expression because *DNMT1* functions to maintain DNA methylation. The impact of EVOO on *DNMT* expression is unknown; however, EVOO contains phenolic compounds, including decarboxymethyl oleuropein aglycone (Montaño et al. 2016), which reduce DNMT activity via competitive inhibition (Corominas-Faja et al. 2018). The absence of a measure of DNMT activity is a limitation of the present study; however, parallel changes in DNMT mRNA expression and activity have previously been reported (Casillas et al. 2003). A measure of activity could potentially explain the lack of association between altered *DNMT* mRNA expression and modulated DNA methylation following supplementation which should be considered in future studies.

The use of a homogenous population of trained cyclists in the present study potentially limits the generalisability of the results to other populations. Trained male cyclists were selected as the population for the present study because they are the most familiar with the exercise stimuli and we would expect this to reflect in the smallest epigenetic response. Previously it has been demonstrated a single bout of exercise was sufficient to reduce global DNA methylation in plasma of COPD patients; however, following a training intervention the exercise bout was no longer sufficient to reduce global DNA methylation (da Silva et al. 2017). Exercise training has previously been demonstrated to alter DNA methylation patterns differently depending on family history of diabetes (Nitert et al. 2012). Future studies should compare the impact of exercise in trained athletes and sedentary individuals or a disease cohort to determine whether exercise-induced alterations to the DNA methylome are contributors to health and disease in diverse populations.

6.5 Conclusion

In conclusion, the present study highlights the impact of an acute bout of aerobic exercise and the supplementation of FAs on DNA methylation and mRNA expression in leukocytes of trained male cyclists. Alterations in the epigenetic control of these genes are associated with physiological markers related to exercise performance and inflammation / oxidative stress; however, a more extensive study is required to confirm these associations. The observational nature of the present study prevents the identification of the underlying mechanisms controlling altered DNA methylation following exercise and FA supplementation; therefore, future mechanistic studies are required to identify such mechanisms. Here we suggest that modulation of DNMT mRNA expression may be one such mechanism for future research; however, future studies should also consider the activity and not just the mRNA expressin of DNMTs. Future studies should also compare multiple tissue types to examine whether exercise and supplementation of FAs have systemic effects on DNA methylation.

Chapter 7 The impact acute and chronic resistance exercise and fatty acid supplementation on global and gene-specific DNA methylation

7.1 Introduction

In the previous two chapters, we have used leukocytes to investigate the impact of an acute bout of aerobic exercise and n-3 PUFA supplementation on DNA methylation. In Chapter 5 we failed to determine any change in DNA methylation; however, the alternative exercise stimuli provided (change in intensity and duration) in Chapter 6 induced significant reductions in global DNA methylation and *PPARGC1A* DNA methylation indicating that the response to exercise is dependent on the type of exercise performed. In the present chapter, we sought to elucidate the impact of acute resistance exercise on DNA methylation to compare the response to an alternative mode of exercise for which limited literature exists and determine whether a chronic training period alters the acute DNA methylation response to exercise.

While, exercise training is known to reduce systemic inflammation (Beavers, Brinkley, and Nicklas 2010; Flynn, McFarlin, and Markofski 2007; Gleeson et al. 2011), the relationship with acute resistance exercise is more complicated. Acute resistance exercise, particularly in individuals who are unaccustomed to the stimulus, causes local muscle damage to the working muscles. Peak muscle damage occurs during eccentric contractions when force is applied to the muscle during the lengthening phase disrupting individual sarcomeres (Peake et al. 2005; Proske and Morgan 2001). Exercise-induced muscle damage is characterised by increased circulating concentrations of intramuscular proteins, including Creatine kinase (CK), Lactate dehydrogenase (LDH) and Myoglobin (Mb); and cytokines, including IL-6 and TNF-α (Brancaccio, Lippi, and Maffulli 2010; Clarkson and Hubal 2002). The response to this muscle damage stimuli is the infiltration of leukocytes into the damaged muscle which further attracts macrophages to remove damaged fibres and leads to the release of various growth factors which regulate satellite cell proliferation differentiation (Tidball 2005).

The majority of literature investigating the impact of exercise on DNA methylation has focussed on the impact of aerobic training (Nitert *et al.*, 2012; Rönn *et al.*, 2013; Rowlands *et al.*, 2014; Denham, *et al.*, 2015; Denham *et al.*, 2015; Lindholm *et al.*, 2015; King-Himmelreich *et al.*, 2016) and acute bouts of aerobic exercise (Barrès et al. 2012; Lane et al. 2015; Robson-Ansley et al. 2014; da Silva et al. 2017); whereas,

limited literature exists epigenetic consequence of acute (Seaborne et al. 2018) and chronic (Denham et al. 2016; Rowlands et al. 2014; Seaborne et al. 2018) resistance exercise. Only one study has compared the impact of both modes of exercise. Rowlands et al. (2014) determined the methylome response to these exercise stimuli regulated different molecular pathways (Rowlands et al. 2014). The mode-specific regulation of the methylome is potentially expected considering aerobic and resistance exercise elicit vastly different adaptations (Coffey and Hawley 2017). Aerobic exercise results in mitochondrial biogenesis and fast to slow fibre type transformation (Hawley 2002; Zierath and Hawley 2004); whereas, resistance exercise stimulates the synthesis of myofibrillar proteins inducing muscle hypertrophy (Damas et al. 2015). The molecular mechanisms controlling the adaption to the different modes of exercise remains to be fully elucidated.

Although the adaptations to aerobic and resistance exercise are diverse, some key molecular signals are witnessed following both modes of exercise. PGC-1α is one of the critical signalling molecules to aerobic exercise and is associated with increased mitochondrial biogenesis (Ventura-Clapier et al. 2008). Previous studies have focussed on the impact of aerobic exercise on the DNA methylation of *PPARGC1A* and other genes related to mitochondrial biogenesis (Alibegovic et al. 2010; Bajpeyi et al. 2017; Barrès et al. 2012; Lund et al. 2017). Increased mRNA expression of *PPARGC1A* following resistance exercise is reported (Ruas et al. 2012; Silvennoinen et al. 2015); however the impact of resistance exercise on *PPARGC1A* DNA methylation is unknown. Aerobic and resistance exercise results in the expression of different isoforms of *PPARGC1A* (Popov et al. 2015; Ruas et al. 2012; Silvennoinen et al. 2015). Aerobic exercise increases the expression from exon 1a via the canonical promoter; whereas, the expression following resistance exercise occurs primarily from the alternative promoter (*PPARGC1A* ALT), situated ~14 kb upstream, known as exon 1b derived *PPARGC1A* (Ruas et al. 2012; Silvennoinen et al. 2015). Increased expression of exon 1b *PPARGC1A* is associated with a hypertrophic response by modulating the expression of insulin-like growth factor-1 and myostatin (Ruas et al. 2012). The mechanisms controlling the exercise mode-specific isoform of *PPARGC1A* remains to be elucidated. DNA methylation has been demonstrated to control promoter usage (Dyrvig et al. 2017), potentially suggesting DNA methylation of these

two promoter regions may explain the exercise-induced differential expression of *PPARGC1A*.

Increased expression of PGC-1α is thought to be an adaption to regular exercise which has an anti-inflammatory consequence (Eisele et al. 2015; Handschin and Spiegelman 2008; Schnyder and Handschin 2015). PGC-1α reduces the activity of the nuclear factor κB, which is known as the master regulator of pro-inflammatory gene expression including the cytokines IL-6 and TNF-α (Eisele et al. 2013). Conversely, sedentary behaviour and gene ablation of PGC-1α is associated with a systemic inflammatory response including increased expression of IL-6 and TNF-α (Handschin and Spiegelman 2008). Elevated systemic levels of inflammation are associated with skeletal muscle atrophy and various myopathies (Muñoz-Cánoves et al. 2013; Reid and Li 2001; Sharples, Al-Shanti, and Stewart 2010), potentially via the hypermethylation of *MyoD* (Sharples et al. 2016). Conversely, the acute local expression of IL-6 and TNF-α is thought to be essential for the repair, regeneration and hypertrophy following muscle-damaging exercise (Chen, Jin, and Li 2007; Li 2013; Muñoz-Cánoves et al. 2013). It has been demonstrated that resistance exercise is sufficient to alter the mRNA expression of both *IL6* and *TNF* in skeletal muscle, but not blood (Gjevestad et al. 2017) indicating transcriptional changes are potentially a mechanism controlling the local production of cytokines; however, a lack of literature exists on the DNA methylation of these critical cytokines in response to muscle-damaging exercise.

The supplementation of the diet with n-3 PUFAs has been demonstrated with an anti-inflammatory phenotype and reduce the levels of inflammatory cytokines (Calder 2015; Tartibian et al. 2011; Vedin et al. 2008, 2012), partially as a result of altered DNA methylation (Aslibekyan et al. 2014; Ma et al. 2016). Supplementation of n-3 PUFAs has also been demonstrated to sensitise skeletal muscle to the anabolic stimuli of resistance exercise and protein ingestion (Philpott et al. 2018; Tachtsis et al. 2018). An anabolic role for n-3 PUFAs is suggested by increases in force production following n-3 PUFA supplementation compared to a control supplementation of corn oil (Smith et al. 2015) and a training only group (Rodacki et al. 2012). A potential epigenetic consequence for the anabolic role of n-3 PUFAs is highlighted by the administration of EPA to skeletal muscle cells can increase the expression of *PPARGC1A* (Tachtsis

et al. 2018) and dampen the effects of TNF-α (Saini et al. 2017), resulting in improved expression of *MyoD* and *Myogenin* indicating increased skeletal muscle differentiation (Saini et al. 2017). Considering the previously discussed impact of *TNF* on hypermethylation of *MyoD*, this suggests a potential mechanism of n-3 PUFAs to reverse epigenetic changes associated with inflammation in skeletal muscle.

Aim

The primary aim of this chapter is to investigate the impact of acute resistance exercise on DNA methylation in leukocytes and skeletal muscle from individuals unaccustomed to resistance exercise. Further, we sought to elucidate whether the FA supplementation or resistance training can modulate acute exercise response.

Objectives

1. Assess whether resistance exercise and FA is sufficient to alter global DNA methylation and mRNA expression of DNMT enzymes.
2. Determine any changes in gene-specific methylation (*PPARGC1A*, *IL6* and *TNF*) and subsequently mRNA expression as a result of exercise and FA supplementation in both skeletal muscle and leukocytes.
3. Determine whether physiological variables of exercise performance, inflammation and markers of muscle damage are associated with DNA methylation.
4. Compare the methylation profiles of leukocytes and skeletal muscle in response to exercise to determine the potential of a tissue specific response.

7.2 Methods

The experimental procedures for this study were approved by the Loughborough University Ethics Human Participants sub-committee (Study ID: R15-P124).

7.2.1 Participants

Sixteen healthy male participants were recruited to the study according to section 3.1. Eight of the participants only completed the acute phase of the study (Trial A to Trial B) and were not included in the data in this thesis. Participants were excluded if they had undertaken resistance training or n-3 PUFA supplementation in the six months before the start of the study or had a previous history of lower limb injuries which may be exacerbated by the eccentric contractions involved in the study.

7.2.2 Study overview

The study consisted of a familiarisation and three experimental trials. The first two trials (Trial A and Trial B) were separated by a three-week double-blind supplementation of either n-3 PUFA or EVOO. Between Trials B and C, participants completed an eight-week eccentric training program of the knee extensors while continuing FA supplementation (Figure 7.1A).

7.2.2.1 Familiarisation

Participants attended a familiarisation session where they underwent anthropometric assessment for height and body mass (section 3.2.1). The Humac Norm dynamometer (CSMI, USA) was then positioned to fit the participant, ensuring the rotational axis of the lever arm was in alignment with the lateral epicondyle of the femur and a hip angle of 85°. The lever arm pad was secured proximal to the malleolus, and participants were secured using stabilising straps to minimise compensatory trunk and thigh movements during testing. Dynamometer settings were recorded to ensure that participants were seated the same during each trial. Participants were then familiarised to the exercise protocols contained within the experimental trials including the performance test (section 7.2.2.3) and muscle damage protocol (section 7.2.2.4)

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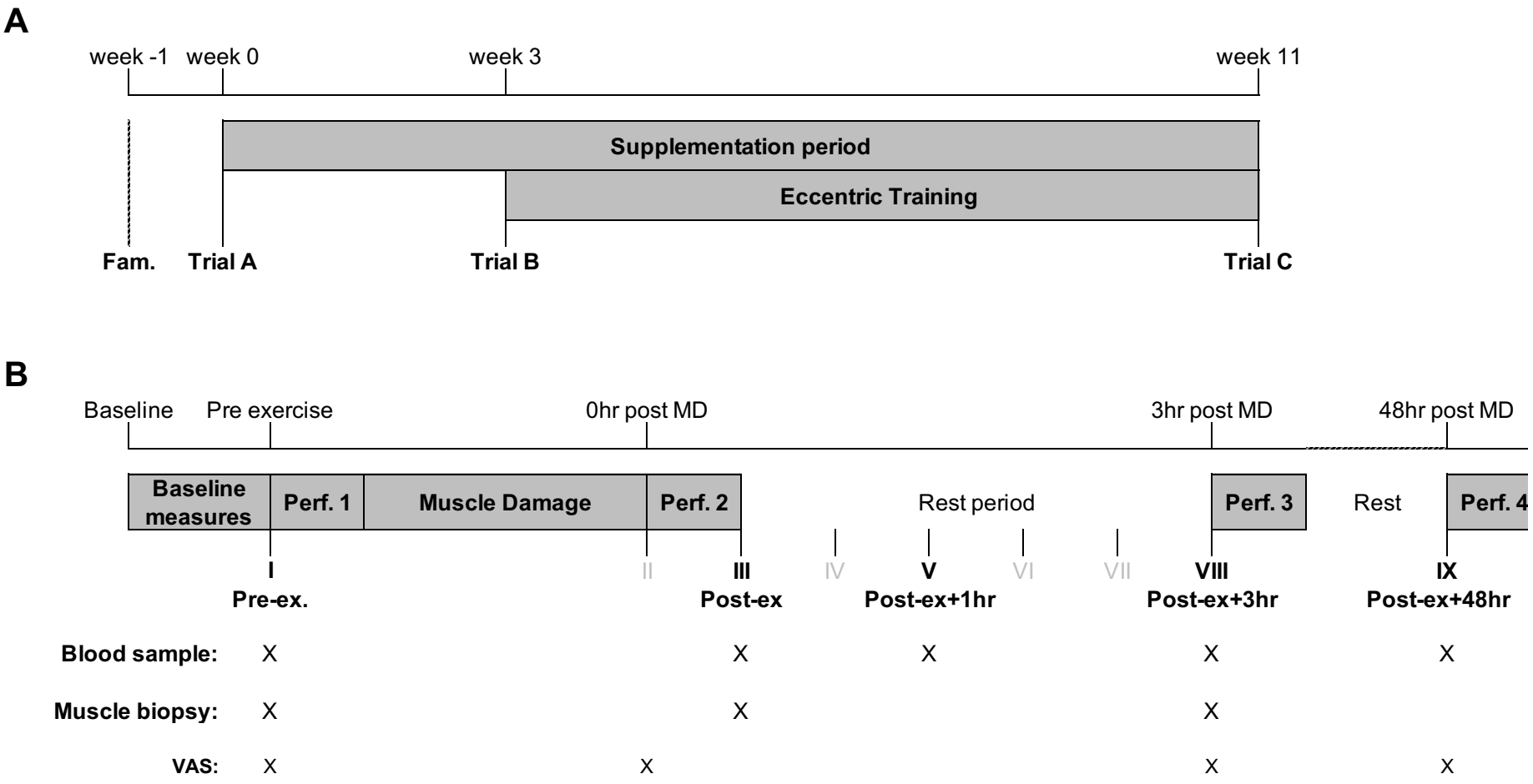

Figure 7.1 – *Schematic representation of (A) study outline and (B) trial day. Roman numerals indicate timepoints where blood samples were collected; however, only time points in black were analysed as part of this thesis. The collection of blood and skeletal muscle tissue, and the collection of VAS data indicated by X. Following completion of performance test 3, participants were free to leave the laboratory and returned 30min before performance test 4 (48hr post-MD). MD, muscle-damaging exercise; Perf., Performance test; VAS, visual analogue scale.*

7.2.2.2 Experimental trials

Figure 7.1B provides a schematic representation of the experimental trials. For each experimental trial, participants reported to the laboratory at the same time of the morning in a fasted and rested state following completion of pre-trial standardisation of diet and exercise (section 3.2.2). On arrival to the laboratory, an intravenous catheter was inserted for the collection of blood samples (section 3.3). Following the collection of baseline biological samples (muscle and blood; section 7.2.3.1) and baseline measures were performed (section 3.2.1), participants completed a performance test (section 7.2.2.3) followed by the muscle damage protocol (section 7.2.2.4). Further performance tests were completed immediately post-exercise (Post-ex), 3hr post-exercise (Post-ex+3hr) and 48hr post-exercise (Post-ex+48hr; Figure 7.1B). The intravenous cannula was removed after completion of the performance test 3hr post muscle-damaging exercise and participants were free to leave the laboratory. Participants returned to the laboratory 30 min before the Post-ex+48hr performance test to allow collection of blood samples via venepuncture (section 3.3).

7.2.2.3 Performance test

Before each performance test participants were asked to indicate the level of muscle soreness using a visual analogue scale (VAS). When seated, participants were asked to extend both legs fully and indicate their perceived level of pain by drawing a single vertical line on a 100mm line ranging from ‘No pain’ (0mm) to ‘Worst possible pain’ (100mm). Participants then completed a five-minute warm-up on a cycle ergometer (Lode B.V, Netherlands) at 75 W. Warm-up was not performed for the performance test immediately post muscle-damaging exercise.

Participants then completed countermovement jumps (CMJ) using a Quattro-Jump 9290AD force platform (Kistler, Switzerland). Three CMJs were competed, with one min recovery between jumps, and the peak height was recorded. If the peak height was achieved on the final jump, another jump was performed (up to a maximum of five).

Participants then performed bilateral maximal voluntary contractions (MVC) of the knee extensors and flexors using a Humac Norm isokinetic dynamometer (CSMI,

USA). Once positioned on the dynamometer, participants performed isometric MVCs of the knee extensors, followed by concentric and eccentric isokinetic MVCs of the knee extensors and flexors. Before each set of MVCs, a warm-up of submaximal contractions (2 x 50%, 1 x 75% and 1 x 90% of perceived MVC) was performed. Thirty seconds rest were provided between submaximal efforts.

For evaluation of isometric torque, three 3s isometric contractions of the knee extensors were performed at 75° of knee flexion (0° = dynamometer lever arm is parallel to the ground). Both concentric and eccentric torque of the knee extensors and flexors was assessed using isokinetic contractions at an angular velocity of 60°/s and a range of motion between 10° and 90° of knee flexion. Verbal encouragement and visual feedback (torque output) were provided for each MVC. A rest period of 60s was provided between each MVC. The highest peak torque obtained during the MVCs were recorded and used for analysis.

7.2.2.4 Eccentric muscle damage protocol

The eccentric muscle damage protocol was performed on the Humac Norm isokinetic dynamometer. The protocol consisted of 20 sets of bilateral maximal voluntary isokinetic eccentric contractions of the knee extensors at an angular velocity of 60°/s using a range of motion between 10 to 90°. Each set consisted of 10 repetitions and was separated by a one-minute rest period. The participants began with their leg at the start position (10°) and were asked to maximally contract the knee extensors throughout the entire range of motion. Once the lever arm reached 90°, participants were asked to relax their leg and allow the lever arm to return to the start position (avoiding concentric contraction of the knee extensors). Verbal encouragement and visual feedback (torque output and work done) were given throughout the muscle damage protocol. The total work completed during the muscle damage protocol was used for analysis.

7.2.2.3 Supplementation

Participants were assigned to either double-blind n-3 PUFA or EVOO supplementation on the basis of age, body mass and preliminary force measurements during Trial A. Both n-3 PUFA (Norwegian Pure-3 AS, Norway) and EVOO supplements were

provided in capsule form following the completion of Trial A. Participants were instructed to consume six capsules per day providing 5.1g of n-3 PUFA (3.0g of EPA, 1.2g of DHA and 0.9g of DPA and other n-3 PUFAs) or 6g of EVOO per day for the entirety of the study (11 weeks). The dose was chosen based on previous findings showing a similar dose was sufficient to induce changes to the FA profile of both blood and skeletal muscle (McGlory et al. 2014). Returned capsules were counted to determine the compliance of supplementation.

7.2.2.4 Eccentric training

In the eight-week period between Trial B and Trial C (Figure 7.1), participants completed an eccentric isokinetic training programme of the knee extensors. Two training sessions were performed per week (14 sessions in total) with a minimum of three days between training sessions. The first training session was completed three days following the end of Trial B, and the last training session was performed three days prior to Trial C. The protocol for the eccentric training programme was the same as the eccentric muscle damage protocol, however the number of sets performed in each session increased throughout the training programme (Table 7.1). The peak force and mean work done per set were recorded in each training session and used for analysis.

Table 7.1 - *Eccentric training programme*

Training session number	Number of sets performed
1 – 2	3
3 – 6	4
7 – 10	5
11 – 14	6

7.2.3 Analytical Procedures

7.2.3.1 Collection of biological tissues

Venous blood samples were collected Pre-ex, Post-ex, Post-ex+1hr, Post-ex+3hr and Post-ex+3hr (Figure 7.1) in each of the three trials (Trial A, Trial B and Trial C). Venous blood was collected (section 3.3) and processed (section 3.4.1) for analysis of DNA methylation, mRNA expression, markers of muscle damage and inflammatory cytokines.

Alongside the collection of blood samples, muscle biopsies were obtained at Pre-ex, Post-ex and Post-ex+3hr (Figure 7.1) for 6 of the 8 participants. Muscle biopsies were obtained from the lateral portion of the *vastus lateralis*. The site was cleaned before an incision into the skin and fascia was made under local anaesthetic (1% Lidocaine). A Bergström biopsy needle with suction was inserted into the incision to extract skeletal muscle tissue. Muscle samples were blotted dry, and any visible fat or connective tissue was removed. Following collection, muscle samples were divided before being snap-frozen in liquid nitrogen and stored at -80˚C prior to DNA and RNA extraction (section 3.4.1).

7.2.3.2 Analysis of DNA methylation

Genomic DNA was extracted from whole blood (section 3.4.1). The concentration of isolated gDNA was 87.5 (± 33.70) ng/uL and an $A_{260/280}$ ratio of 1.89 (± 0.02). LUMA was performed to determine global DNA methylation (section 3.4.2). For determination of gene-specific DNA methylation, DNA was extracted and bisulfite-converted from whole blood and skeletal muscle using the EPITect Fast LyseAll Bisulfite kit (section 3.4.1). Bisulfite-converted DNA underwent PCR using the Pyromark PCR kit (section 3.4.3). DNA methylation percentage was then determined via pyrosequencing (section 3.4.3). The assays used to determine DNA methylation are presented in Table 4.2 (*PPARGC1A, PPARGC1A ALT, IL6 and TNF*).

7.2.3.3 Analysis of mRNA expression

RNA was extracted from whole blood using TRIzol LS and skeletal muscle using TRI Reagent (section 3.4.5). The concentration of RNA isolated from whole blood was 55.36 (± 16.60) ng/uL and an A_{260}/A_{280} ratio of 1.97 (± 0.05), whereas, the

concentration of RNA isolated from skeletal muscle was 403.34 (± 151.99) ng/uL and an A_{260}/A_{280} ratio of 2.04 (± 0.03).

RNA was then cDNA converted (section 3.4.5), and relative mRNA expression was performed using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001) using GAPDH as the reference gene (section 3.4.6). Primer sequences for the assays used to determine mRNA expression of the genes of interest are displayed in Table 4.3 (*GAPDH, PPARGC1A Total, PPARGC1A* Exon1a, *PPARGC1A* Exon 1b, *IL6, DNMT1, DNMT3a* and *DNMT3b*). The mean Ct value for GAPDH (reference gene) was consistent across all participants and experimental conditions in whole blood (17.31 ± 0.725) and skeletal muscle (12.89 ± 0.475) with a low variation of 4.18% and 3.681% respectively.

7.2.3.4 Cytokine analysis

Serum isolated from whole blood collected into silica coated vacutainers (section 3.3) at time points Pre-ex, Post-ex, Post-ex+3hr and Post-ex+48hr in each trial. The serum was then used to determine the circulating levels of IL-6 and TNF-α using BD™ Cytometric Bead Array Enhanced Sensitivity Flex Sets (BD Bioscience, UK) on a flow cytometry platform (BD Accuri™ C6 Flow Cytometer, BD Bioscience, UK). All serum samples were diluted 1:3 with CBA buffer. All samples for a participant were performed within a single run to minimise run-to-run variation. Haematocrit and haemoglobin values were used to ascertain plasma volume changes that were used to adjust serum IL-6 and TNF-α concentrations (Dill and Costill 1974).

7.2.3.5 Markers of muscle damage

ABX pentra assays (Horiba Medical, Japan) were used to determine serum concentrations of creatine kinase (CK), lactate dehydrogenase (LDH) and myoglobin (Mb) using a Pentra C400 analyser (Horiba Medical, Japan) at the time points Pre-ex, Post-ex, Post-ex+3hr and Post-ex+48hr in each trial. All samples for a participant were performed within a single run to minimise run-to-run variation. Haematocrit and haemoglobin values were used to ascertain plasma volume changes that were used to adjust serum CK, LDH and Mb concentrations (Dill and Costill 1974).

7.2.4 Statistical Analysis

Statistical analysis was performed according to section 3.5. Differences between the supplementation groups for physiological variables (anthropometrics, measures of muscle strength, and DNA methylation) at baseline (Trial A Pre-ex) were investigated using t-tests. DNA methylation, mRNA expression values and physiological variables related to exercise performance, inflammation and muscle damage were analysed using a 3-way between (Supplement) x within (Trial) x within (Time) RM-ANOVA. The work done during the muscle damage protocol and the change in performance during the training programme was analysed using a 2-way between (Supplement) x within (Trial / Session) RM-ANOVA.

Spearman's Rho correlation analysis was used to assess the relationship between DNA methylation and mRNA expression, and physiological markers related to exercise performance, inflammation and muscle damage. Moderate (>0.5) correlation coefficients were considered to be of interest; however, only large (> 0.7) correlation coefficients were deemed statistically significant. All data presented as mean ± 95% CI unless otherwise stated.

7.3 Results

7.3.1 Baseline measurements

Participant characteristics are presented in Table 7.2. No differences between the groups were detected for baseline (Trial A, Pre-ex) anthropometric or exercise performance measures ($p > 0.05$; Table 7.2). Baseline DNA methylation values were compared between supplement groups, and no differences were detected for global, *PPARGC1A*, *PPARGC1A* ALT or *TNF* DNA methylation ($p > 0.05$; Table 7.3. Leukocyte DNA methylation of 3 CpG sites in the *IL6* promoter were significantly higher in the EVOO group ($p < 0.05$; Table 7.3). A non-significant trend for increased serum IL-6 concentrations in the EVOO group was also identified at baseline (Trial A, Pre-ex). The levels of DNA methylation were significantly different between tissues; DNA methylation was significantly higher in leukocytes for each CpG site of *PPARGC1A*, *PPARGC1A* ALT and *IL6*; whereas, the DNA methylation of *TNF* was increased in skeletal muscle ($p < 0.01$; Table 7.3).

Table 7.2- *Participant characteristics of the overall cohort and the n-3 PUFA and EVOO supplement group. Differences between groups for anthropometric and exercise performance measures assessed using paired T-tests. Values are means ± standard deviation*

Variable	Overall (n = 8)	n-3 PUFA (n = 4)	EVOO (n = 4)	p-value
Anthropometrics				
Age (yrs)	27.98 ± 7.19	30.21 ± 10.31	25.75 ± 0.91	0.601
Height (cm)	177.76 ± 4.92	176.05 ± 5.04	179.48 ± 4.83	0.321
Body mass (kg)	83.15 ± 17.33	80.25 ± 18.74	86.05 ± 18.09	0.962
BMI ($kg \cdot m^{-2}$)	26.19 ± 4.54	25.71 ± 4.64	26.67 ± 5.09	0.731
Exercise performance				
Peak isometric torque (Nm)	251.38 ± 49.19	234 ± 60.27	268.75 ± 34.76	0.421
Peak concentric torque (Nm)	204.44 ± 47.4	197.25 ± 66.13	211.63 ± 27.05	0.725
Peak eccentric torque (Nm)	271.69 ± 59.92	248.38 ± 56.54	295 ± 61.09	0.441
CMJ jump height (cm)	39.43 ± 9.86	37.68 ± 4.25	41.18 ± 14.16	0.653

Table 7.3 - *Methylation of skeletal muscle and leukocytes at baseline (Trial A, Pre-ex) in the whole cohort and the each supplement group. § indicates a difference (p < 0.01) in methylation between skeletal muscle and leukocytes overall. p-value <0.05 indicates a difference between supplement groups. n-3 PUFA, omega-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil. Data presented as mean ± SD*

CpG Site		Overall	n-3 PUFA	EVOO	p-value
Global methylation					
LUMA	Leukocytes	80.05 ± 0.42	79.95 ± 0.61	80.15 ± 0.17	0.550
PPARGC1A					
CpG1	Skeletal Muscle	3.55 ± 0.96	3.99 ± 1.09	3.12 ± 0.84	0.335
	Blood	8.53 ± 2.77§	7.39 ± 0.41	9.68 ± 3.77	0.272
***PPARGC1A* ALT**					
CpG1	Skeletal Muscle	16.82 ± 3.49	17.71 ± 2.72	15.93 ± 4.25	0.574
	Blood	62.31 ± 3.43§	61.95 ± 3.7	62.66 ± 3.66	0.796
CpG2	Skeletal Muscle	5.12 ± 2.47	5.55 ± 2.92	4.68 ± 2.01	0.694
	Blood	62.70 ± 1.78§	62.35 ± 2.44	63.05 ± 1.05	0.618
CpG3	Skeletal Muscle	7.35 ± 3.61	7.55 ± 3.54	7.15 ± 3.68	0.897
	Blood	78.82 ± 1.71§	79.11 ± 1.94	78.53 ± 1.67	0.671
CpG Mean	Skeletal Muscle	9.76 ± 3.17	10.27 ± 3.03	9.25 ± 3.31	0.715
	Blood	67.94 ± 1.71§	67.8 ± 2.06	68.08 ± 1.60	0.839
IL6					
CpG1	Skeletal Muscle	72.41 ± 6.93	73.92 ± 4.27	70.9 ± 9.58	0.644
	Blood	91.10 ± 1.92§	**89.66 ± 0.51**	**92.53 ± 1.69**	**0.018***
CpG2	Skeletal Muscle	76.87 ± 4.46	77.91 ± 2.91	75.82 ± 6.00	0.615
	Blood	91.12 ± 1.19§	**90.32 ± 1.03**	**91.93 ± 0.72**	**0.043***
CpG3	Skeletal Muscle	82.94 ± 3.97	84.82 ± 1.26	81.06 ± 6.68	0.392
	Blood	91.17 ± 3.09§	90.01 ± 4.31	92.32 ± 0.46	0.327
CpG4	Skeletal Muscle	66.08 ± 5.03	68.57 ± 2.97	63.58 ± 7.09	0.323
	Blood	88.89 ± 1.95§	89.09 ± 2.74	88.7 ± 1.12	0.804
CpG5	Skeletal Muscle	72.23 ± 4.025	73.27 ± 2.18	71.18 ± 5.87	0.593
	Blood	82.01 ± 3.58§	**79.52 ± 3.61**	**84.5 ± 0.59**	**0.034***
CpG6	Skeletal Muscle	74.09 ± 5.38	75.13 ± 2.89	73.04 ± 7.87	0.689
	Blood	88.99 ± 2.3§	87.87 ± 1.8	90.1 ± 2.4	0.187
CpG Mean	Skeletal Muscle	74.10 ± 4.14	75.61 ± 1.34	72.6 ± 6.94	0.502
	Blood	88.87 ± 1.42§	**87.74 ± 0.94**	**90.01 ± 0.65**	**0.007#**
TNF					
CpG1	Skeletal Muscle	30.82 ± 5.11	32.22 ± 5.49	29.42 ± 4.73	0.541
	Blood	13.17 ± 2.70§	11.88 ± 2.23	14.46 ± 2.76	0.197
CpG2	Skeletal Muscle	25.03 ± 3.88	25.49 ± 3.93	24.58 ± 3.83	0.789
	Blood	10.53 ± 2.5§	9.36 ± 1.65	11.70 ± 2.87	0.207
CpG3	Skeletal Muscle	30.41 ± 3.17	31.07 ± 3.41	29.75 ± 2.93	0.638
	Blood	12.62 ± 3.42§	10.71 ± 3.64	14.53 ± 2.06	0.118
CpG4	Skeletal Muscle	50.87 ± 5.64	51.3 ± 6.39	50.43 ± 4.88	0.860
	Blood	14.65 ± 3.31§	13.17 ± 3.42	16.13 ± 2.84	0.231
CpG Mean	Skeletal Muscle	34.28 ± 4.38	35.02 ± 4.8	33.54 ± 3.96	0.703
	Blood	12.74 ± 2.58§	11.28 ± 2.14	14.2 ± 2.29	0.111

7.3.2 Physiological responses - Exercise performance, inflammation and muscle damage

7.3.2.1 Exercise performance

FA supplementation did not alter the total work done during the muscle damage protocol (Trial A vs Trial B; $p > 0.05$; Figure 7.2A); however, eccentric training increased the work done during the muscle damage protocol ($p < 0.01$). Similarly, increased mean work done per set during the training period was detected regardless of n-3 PUFA or EVOO supplementation (Session 14 vs Session 1; $p < 0.05$; Figure 7.2B).

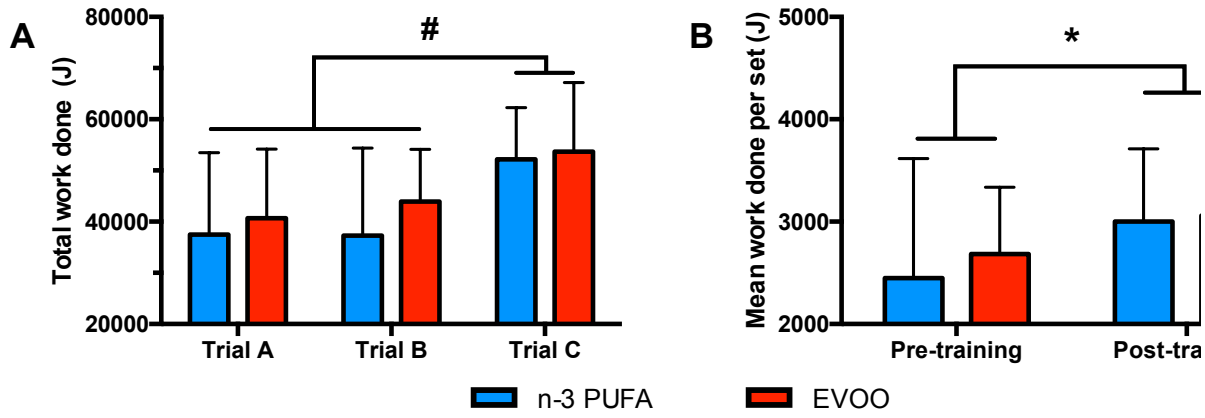

Figure 7.2 - *Impact of fatty acid supplementation and eccentric training on (A) Total work completed during muscle damage protocol during Trial A, Trial B and Trial C, and (B) Mean work completed per set Pre-training (first training session) and Post-training (final training session). n-3 PUFA, omega-3 polyunsaturated fatty acids; EVOO, extra virgin olive oil. *indicates $p < 0.05$, #indicates $p < 0.01$ between time points.*

No impact of FA supplementation was detected for measures of exercise performance during the performance tests ($p > 0.05$). A main effect of Time was identified independent of Supplement and Trial indicating a reduction in CMJ height Post-ex which remained significant until Post-ex+48hr ($p < 0.05$; Figure 7.3A). Isometric peak torque reduced Post-ex, and Post-ex+3hr compared to Pre-ex ($p < 0.01$; Figure 7.3B). The isometric peak torque values were higher in Trial C (Post-training) compared to Trial A indicating the eccentric training increased peak force ($p = 0.027$; Figure 7.3B). Significant interactions were identified between trial and time for the eccentric and concentric force of the knee extensors ($p < 0.05$). Peak force for both eccentric and concentric contractions decreased Post-ex and Post-ex+3hr; however, the reduction

in peak force was attenuated during Trial C compared to Trial A and Trial B ($p < 0.01$; Figure 7.3C & 7.3D).

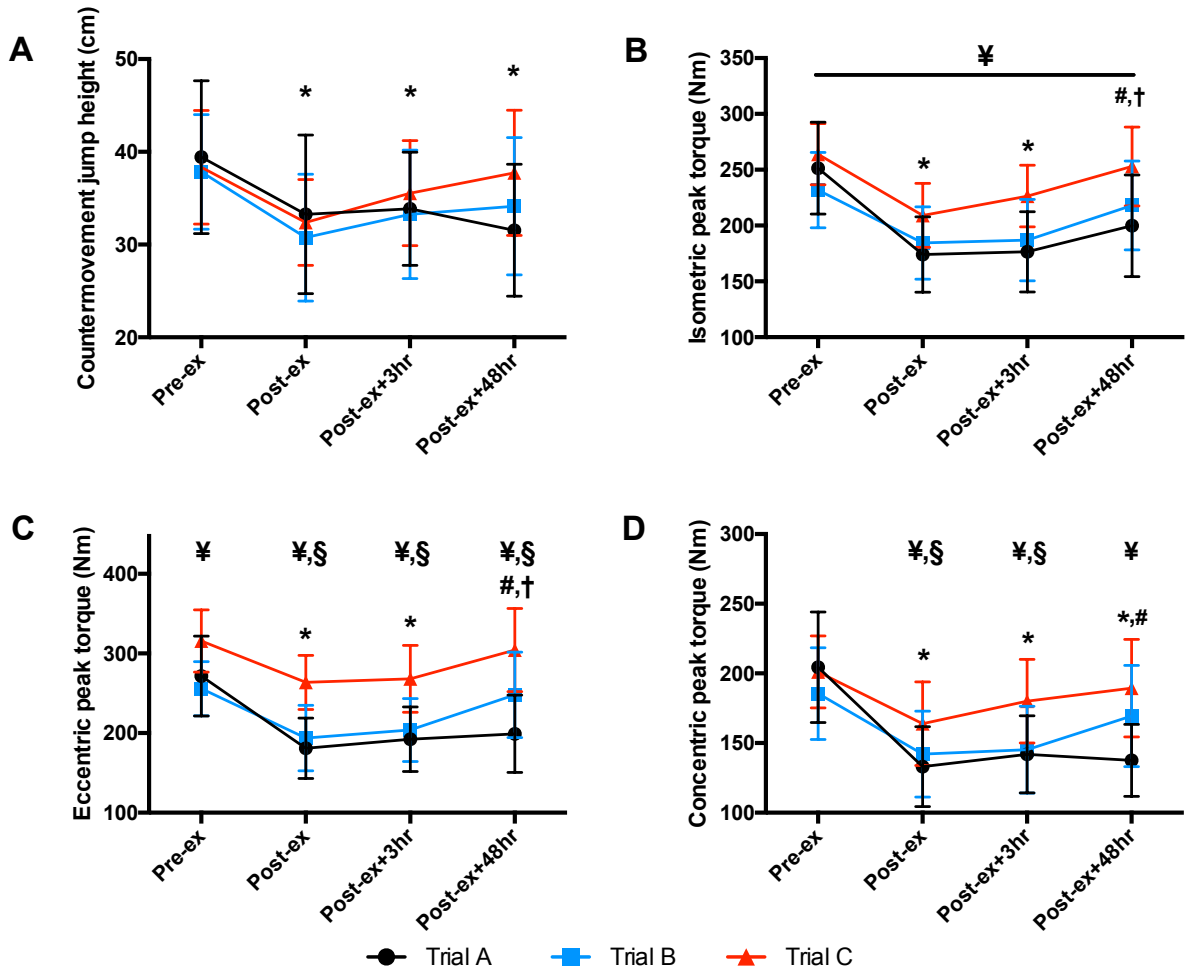

Figure 7.3 *– Impact of exercise on exercise performance; (A) Countermovement jump height, (B) Isometric peak torque, (C) eccentric and (D) concentric peak torque of the knee extensors. *indicates significantly different from Pre-ex; # indicates significantly different from Post-ex; † indicates significantly different from Post-ex+3hr. ¥ indicates a difference between Trial A and Trial C; § indicates a difference between Trial B and Trial C.*

7.3.2.2 Markers of inflammation and muscle damage

A main effect of time identified a significant increase in serum IL-6 concentration Post-ex and Post-ex+3hr ($p < 0.05$; Figure 7.4A); whereas, circulating TNF-α concentrations were unaffected by exercise (Figure 7.4B). Neither FA supplementation or exercise training altered IL-6 or TNF-α concentrations ($p>0.05$).

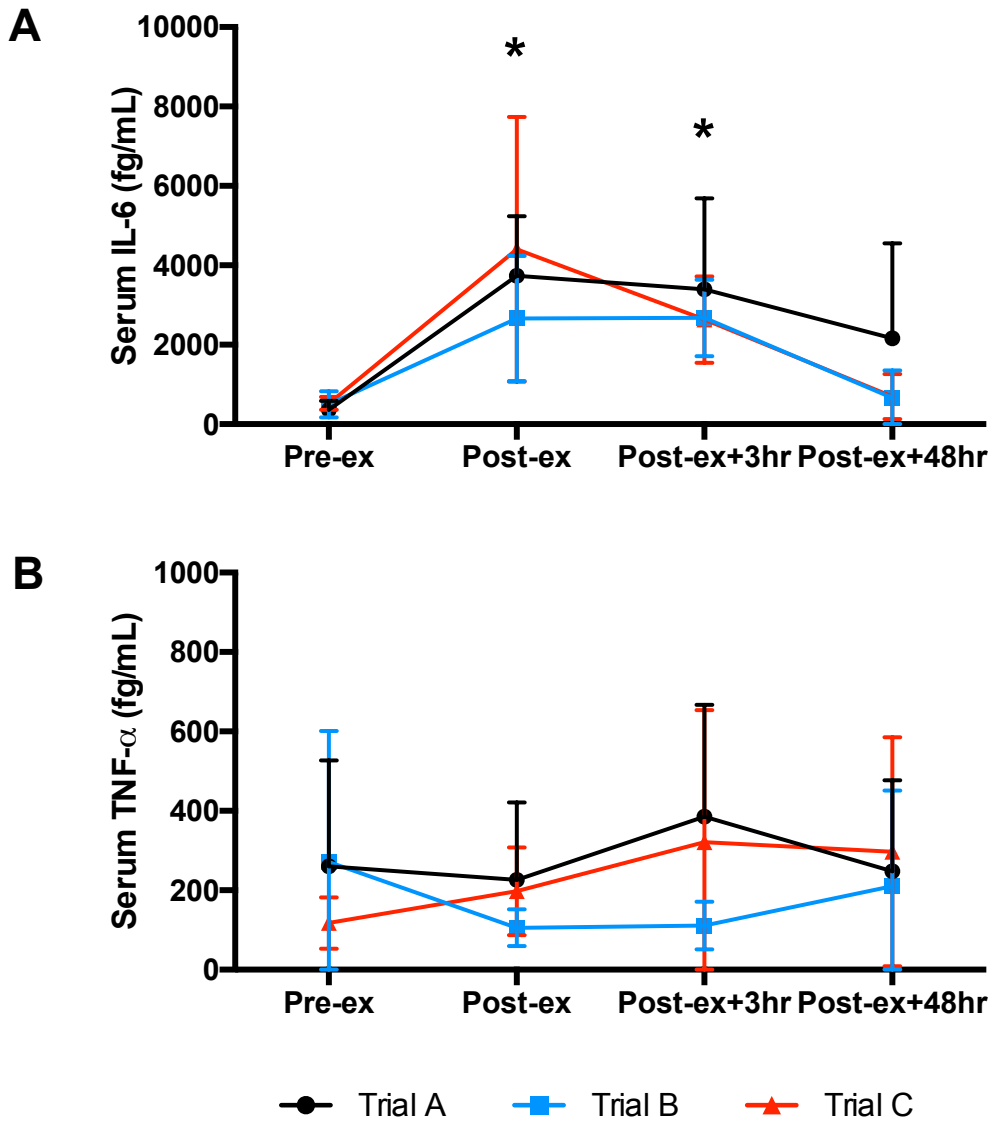

Figure 7.4 *-Serum concentrations of cytokines (A) Interleukin-6 (IL-6) and (B)* TNF-α **indicates significantly different from Pre-ex.*

The serum concentration of the muscle damage markers CK ($p = 0.026$) and MB ($p = 0.002$) were increased following exercise. Compared to the Pre-ex values a significant increase in CK and MB was detected Post-ex and Post-ex+3hr ($p < 0.05$; Figure 7.5). The circulating concentrations of LDH remained unchanged by exercise ($p > 0.05$; Figure 7.5B). FA supplementation and eccentric training did not alter the serum concentrations of the markers of muscle damage ($p > 0.05$); however, non-significant trends for a main effect of trial were detected for CK ($p = 0.052$) and MB ($p = 0.087$), suggesting a potential reduction in protein concentrations with repeated bouts of exercise.

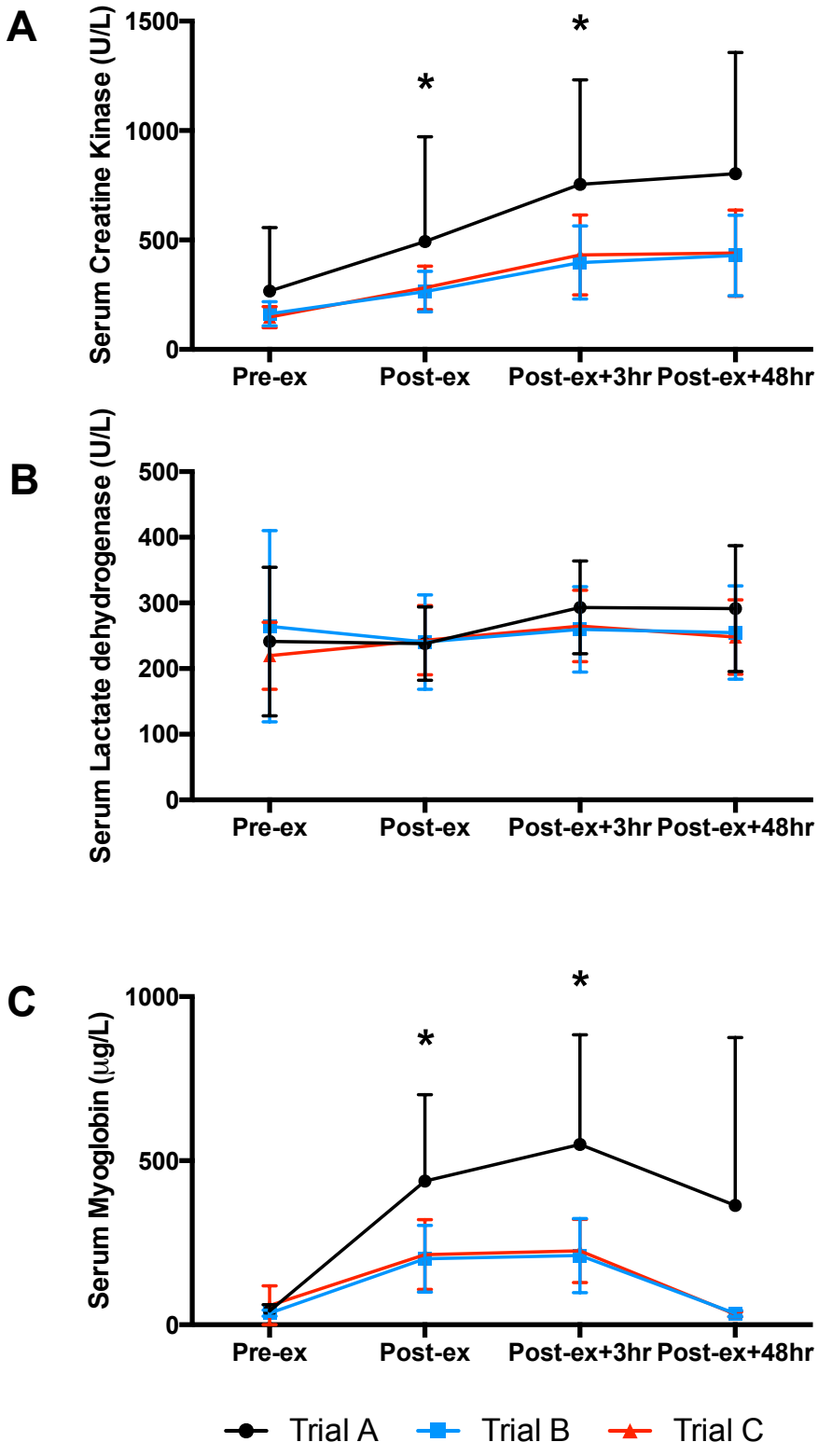

Figure 7.5 -*Serum concentration of markers muscle damage markers (A) Creatine kinase, (B) Lactate dehydrogenase and (C) Myoglobin. *indicates significantly different from Pre-ex.*

7.3.2 Global cytosine methylation and DNMT mRNA expression

While FA supplementation group did not alter global DNA methylation, an interaction between trial and time was identified for global DNA methylation ($p = 0.004$). Pairwise comparisons indicate global methylation in each trial was significantly different at Post-ex+3hr ($p < 0.032$; Figure 7.6).

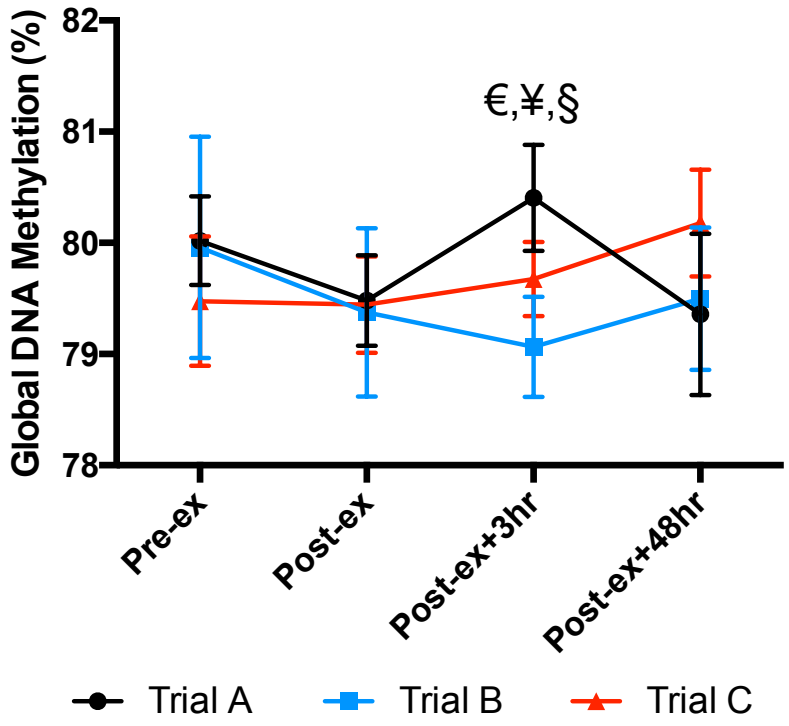

Figure 7.6 - *Effect of exercise on global DNA methylation. € indicates a difference between Trial A and Trial B; ¥ indicates a difference between Trial A and C; § indicates a difference between Trial B and C.*

A main effect of time was detected in skeletal muscle and leukocytes for the mRNA expression of *DNMT3a* and *DNMT3b* ($p < 0.01$; Figure 7.7) but not *DNMT1* ($p > 0.05$; Figure 7.7A & 7.7B); however, neither supplementation or training altered the mRNA expression of the DNMT enzymes ($p > 0.05$). Skeletal muscle *DNMT3a* mRNA expression was unaltered between Pre-ex and Post-ex ($p = 0.543$); however, a significant decrease in *DNMT3a* mRNA expression was detected Post-ex+3hr compared to both Pre-ex and Post-ex time points ($p < 0.006$; Figure 7.7C). A delayed response was also detected in leukocytes with increased mRNA expression Post-ex+48h compared to Post-ex ($p = 0.037$; Figure 7.7D). An immediate decrease in *DNMT3b* mRNA expression was identified in skeletal muscle and leukocytes, which

remained significant until the Post-ex+3hr time point in both tissues ($p < 0.05$; Figure 7.7). Despite no change in global methylation, significant positive correlations were determined between Post-ex and Post-ex+3hr global methylation and the mRNA expression of both *DNMT3a* and *DNMT3b* ($p < 0.05$; Figure 7.13).

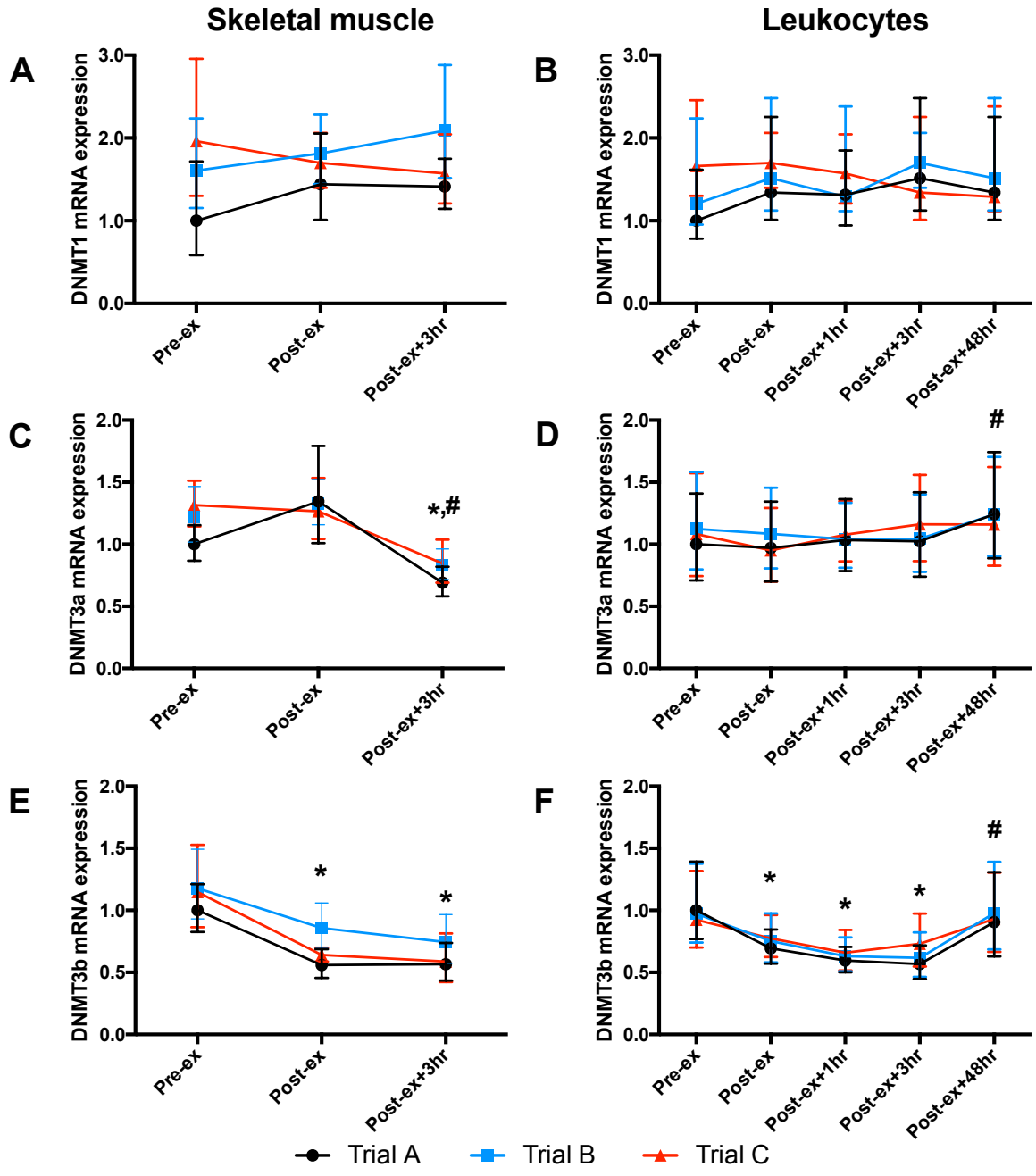

Figure 7.7 - *Effect of exercise on the mRNA expression of (A&B) DNMT1, (C&D) DNMT3a and (E&F) DNMT3b in skeletal muscle (left-hand column) and leukocytes (right-hand column). *indicates significantly different from Pre-ex; # indicates significantly different from Post-ex.*

7.3.3 Gene-specific DNA Methylation and mRNA expression

The supplementation of FA did not alter the DNA methylation or mRNA expression of *PPARGC1A*, *IL6* or *TNF* in either skeletal muscle or leukocytes ($p > 0.05$); therefore, the supplement groups were combined to examine the impact of exercise on DNA methylation and mRNA expression.

7.3.3.1 PPARGC1A

The methylation at the individual CpG sites for the *PPARGC1A* and *PPARGC1A* ALT assays are presented in Table 7.4, whereas the impact of exercise within each trial on the mean methylation across all CpG sites is presented in Figure 7.8.

A significant increase in *PPARGC1A* DNA methylation was detected Post-ex in skeletal muscle ($p = 0.022$), which returned back to Pre-ex values by Post-ex+3hr ($p > 0.05$; Figure 7.8A). A main effect of time was also identified for *PPARGC1A* methylation in leukocytes; however, no significant pairwise comparisons were detected following Bonferroni correction ($p > 0.05$; Table 7.4). Increased DNA methylation of *PPARGC1A* ALT was detected in both skeletal muscle and leukocytes following exercise ($p <0.05$; Figure 7.8). In skeletal muscle, an immediate increase in DNA methylation was determined Post-ex for each CpG site ($p < 0.05$), which did not fully return to baseline values by Post-ex+3hr (Table 7.4). A delayed response was detected for *PPARGC1A* ALT in leukocytes, which became significant at Post-ex+3hr for 2 of the CpG sites (-182 & -127) and the mean methylation across all CpG sites (Table 7.4).

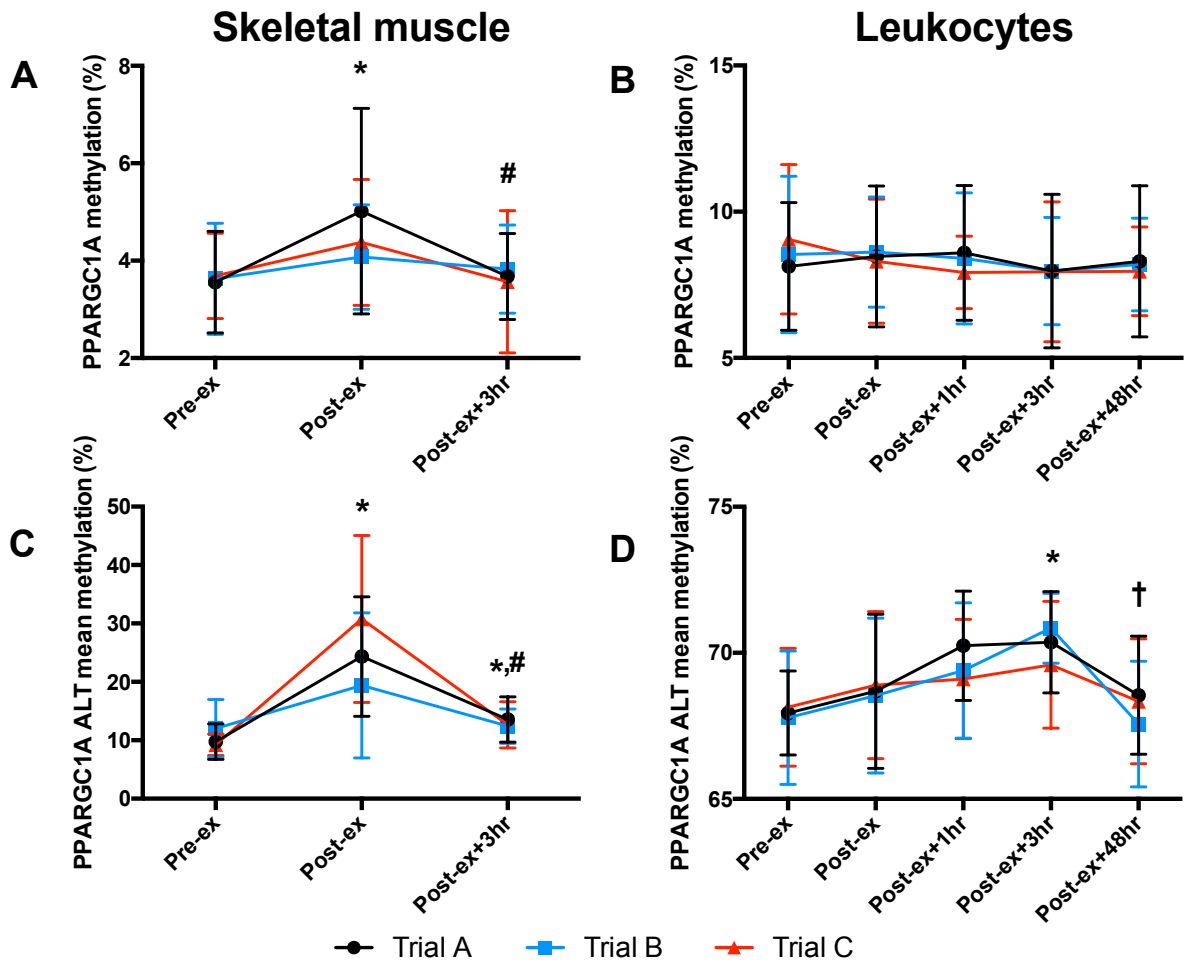

Figure 7.8 - *Effect of exercise on DNA methylation of the mean methylation of the CpG sites analysed by the (A&B) PPARGC1A and (C&D) PPARGC1A ALT assays in skeletal muscle (left-hand column) and leukocytes (right-hand column). *indicates significantly different from Pre-ex; # indicates significantly different from Post-ex; † indicates significantly different from Post-ex+3hr.*

PPARGC1A mRNA expression, in skeletal muscle, was determined using three separate assays to identify the mRNA expression from each of the promoters (Exon 1a and 1b derived) and the overall mRNA expression. Assays to determine expression from the different *PPARGC1A* promoters failed in leukocytes; therefore, only Total *PPARGC1A* mRNA expression is reported in leukocytes (Figure 7.9). In skeletal muscle increased mRNA expression of Exon 1a, Exon 1b and total *PPARGC1A* was detected Post-ex ($p < 0.05$; Figure 7.9A-C). Exon 1a derived *PPARGC1A* mRNA expression returned to Pre-ex values by Post-ex+3hr ($p = 0.855$); whereas, at the Post-ex+3hr time point Exon 1b derived, and Total PAPRGC1A mRNA expression

remained elevated compared to Pre-ex ($p < 0.01$; Figure 7.9). Total *PPARGC1A* mRNA expression was unaltered by exercise in leukocytes ($p > 0.05$; Figure 7.9D).

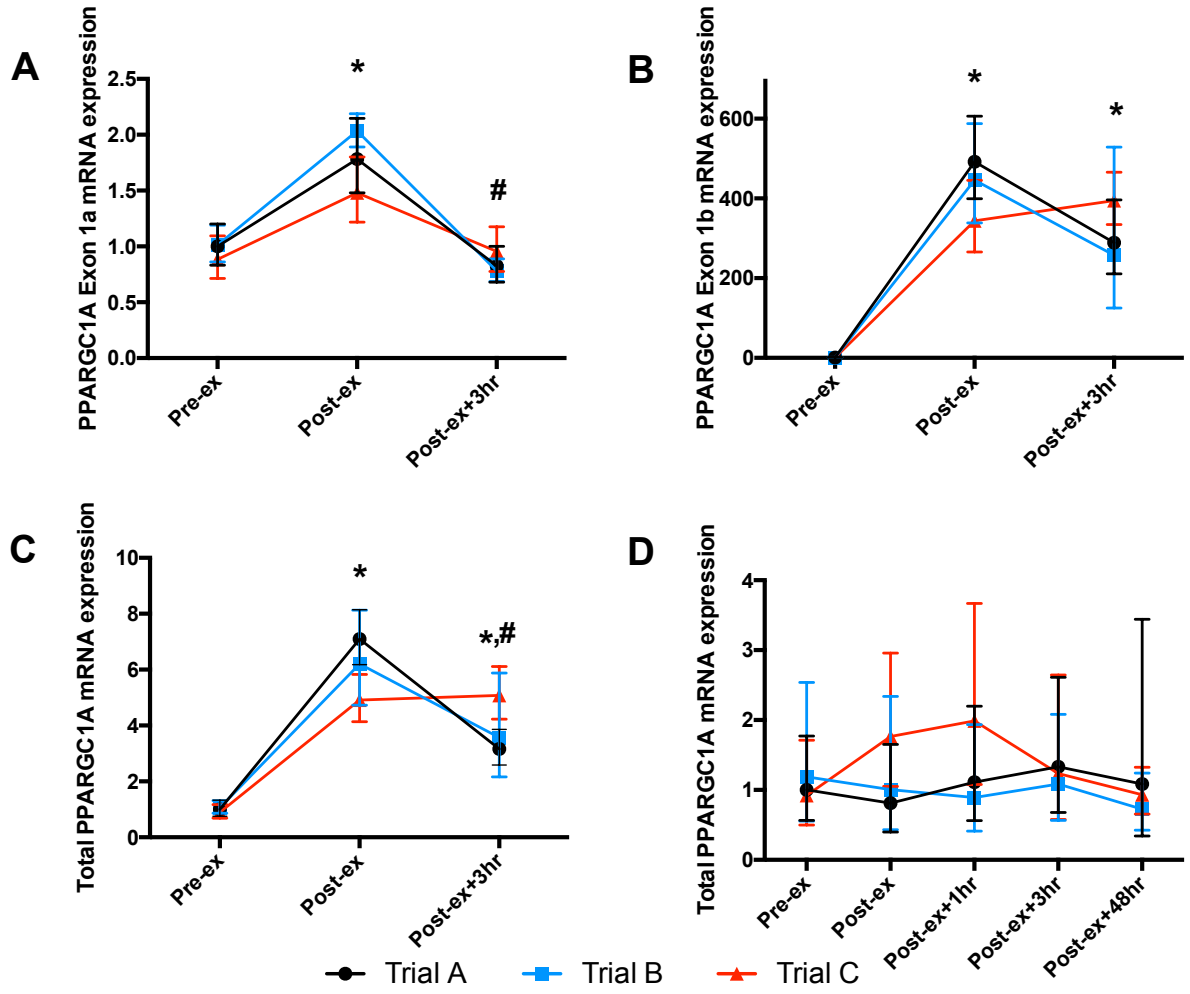

Figure 7.9 – *Impact of exercise on promotor specific PPARGC1A mRNA expression in skeletal muscle (A-C) and leukocytes (D). *indicates significantly different from Pre-ex; # indicates significantly different from Post-ex.*

No significant associations were identified between *PPARGC1A* DNA methylation and PAPRGC1A mRNA expression in either skeletal muscle (Figure 7.12) or leukocytes (Figure 7.13; $p > 0.05$). Post-ex+1hr mRNA expression of *DNMT3b* in leukocytes was positively correlated with Post-ex and Post-ex+3hr *PPARGC1A* methylation ($p < 0.05$; Figure 7.13). In skeletal muscle, *IL6* mRNA expression was negatively correlated with *PPARGC1A* ALT methylation ($p < 0.05$; Figure 7.12); whereas, *PPARGC1A* methylation in leukocytes was positively correlated with *IL6* mRNA expression ($p < 0.05$; Figure 7.13) indicating a role of *IL6* mRNA expression in DNA methylation of both *PPARGC1A* promoters.

Table 7.4 - *DNA methylation of the PPARGC1A and PPARGC1A ALT assays. Data presented as the mean of all three trials ± standard deviation. Values not sharing a letter (a,b,c) are significantly different for simple interactions of time (p<0.05) after Bonferroni correction for multiple testing.*

CpG site	Tissue	Pre-Ex	Post-ex	Post-ex+1hr	Post-ex+3hr	Post-ex+48hr	Main effect p-value
PPARGC1A CpG1: -260	Skeletal muscle	3.62 ± 0.74^{a}	4.49 ± 1.16^{b}	N.D.	3.69 ± 0.80^{a}	N.D.	**0.003**
	Leukocytes	8.95 ± 2.15	8.48 ± 1.83	7.53 ± 1.73	8.05 ± 2.04	8.39 ± 1.78	**0.041**
PPARGC1A ALT CpG1: -182	Skeletal muscle	16.55 ± 2.82^{a}	28.24 ± 8.76^{b}	N.D.	19.24 ± 3.06^{ab}	N.D.	**0.001**
	Leukocytes	62.45 ± 2.61^{a}	63.11 ± 3.25^{a}	64.86 ± 2.3^{ab}	66.03 ± 1.83^{b}	62.28 ± 2.63^{a}	**0.001**
PPARGC1A ALT CpG2: -131	Skeletal muscle	6.01 ± 2.35^{a}	20.29 ± 9.23^{b}	N.D.	8.21 ± 2.31^{c}	N.D.	**0.001**
	Leukocytes	62.79 ± 1.5	63.16 ± 1.68	63.28 ± 1.22	63.59 ± 1.47	63.07 ± 1.38	0.294
PPARGC1A ALT CpG3: -127	Skeletal muscle	8.48 ± 3.26^{a}	25.98 ± 11.23^{b}	N.D.	11.18 ± 2.84^{c}	N.D.	**0.001**
	Leukocytes	78.62 ± 1.48^{a}	79.85 ± 1.99^{a}	80.69 ± 1.84^{ab}	81.18 ± 1.46^{b}	79.1 ± 1.87^{ab}	**0.009**
PPARGC1A ALT CpG: Mean	Skeletal muscle	10.35 ± 2.71^{a}	24.84 ± 9.69^{b}	N.D.	12.88 ± 2.62^{c}	N.D.	**0.001**
	Leukocytes	67.95 ± 1.54^{a}	68.71 ± 2.06^{ab}	69.61 ± 1.57^{ab}	70.26 ± 1.43^{b}	68.15 ± 1.69^{a}	**0.001**

7.3.3.2 IL6

The mean methylation across all conditions at the individual CpG sites for the *IL6* assay are presented in Table 7.5, whereas the impact of exercise within each trial on the mean methylation across all CpG sites are presented in Figure 7.10.

A main effect of time was detected for DNA methylation each of the *IL6* CpG sites and *IL6* mRNA expression in skeletal muscle ($p < 0.025$; Table 7.5). A transient increase in *IL6* methylation was detected Post-ex at each CpG which returned toward Pre-ex values by Post-ex+3hr ($p < 0.05$; Table 7.5). Similarly, in skeletal muscle an immediate increase in *IL6* mRNA expression Post-ex was identified; however, mRNA expression returned to Pre-ex expression levels by Post-ex+3hr ($p < 0.01$; Figure 7.10C). No correlations were determined between the mean methylation across all CpG sites and mRNA expression of *IL6* or the DNMT enzymes ($p > 0.05$; Figure 7.12).

Within leukocytes, a main effect of time was detected for methylation of two CpG sites (CpG2: -1096 & CpG4: -1069; $p < 0.05$; Table 7.5). At CpG2 a delayed increase in methylation was detected Post-ex+3hr, which returned to baseline values by Post-ex+48hr ($p < 0.05$; Table 7.5). A similar non-significant trend ($p = 0.051$) was determined for the methylation across all *IL6* CpG sites analysed, with peak methylation occurring Post-ex+3hr (Table 7.5). Unlike in skeletal muscle, an immediate decrease in methylation was determined Post-ex at *IL6* CpG4 in leukocytes indicating differential response between skeletal muscle and leukocytes ($p < 0.05$; Table 7.5). *IL6* mRNA expression in leukocytes was unaltered by exercise and no consistent correlations were determined between *IL6* DNA methylation and mRNA expression were identified (Figure 7.13).

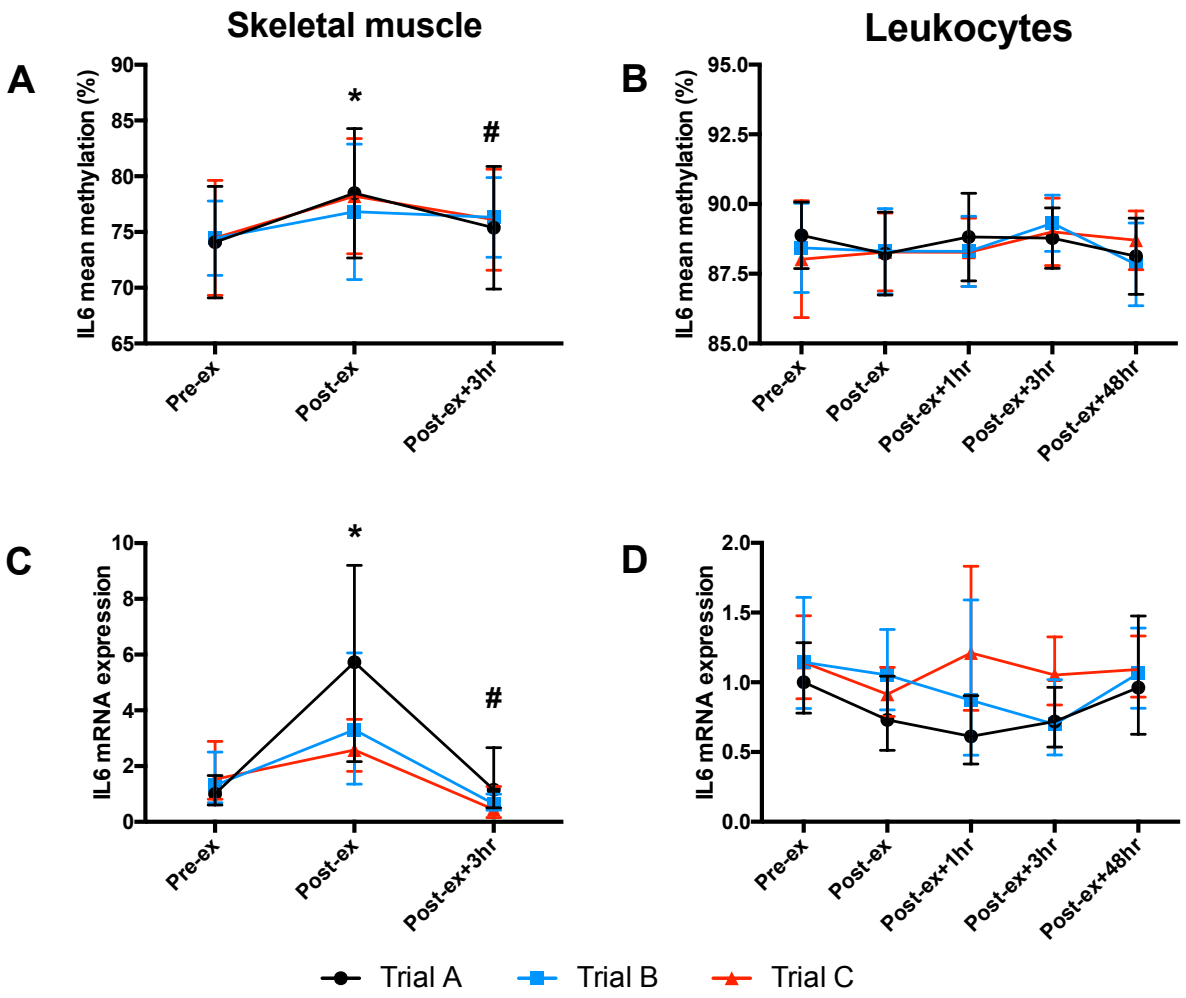

Figure 7.10 - *Impact of exercise on mean IL6 DNA methylation across all CpG sites (A & B) and mRNA expression (C & D) in skeletal muscle (left-hand column) and leukocytes (right-hand column). *indicates significantly different from Pre-ex; # indicates significantly different from Post-ex.*

Table 7.5 - *DNA methylation of IL6. Data presented as the mean of all trials ± standard deviation. Values not sharing a letter (a,b,c) are significantly different for simple interactions of time (p<0.05) after Bonferroni correction for multiple testing.*

CpG site	Tissue	Pre-Ex	Post-ex	Post-ex+1hr	Post-ex+3hr	Post-ex+48hr	Main effect p-value
IL6 CpG1: -1099	Skeletal muscle	73.06 ± 4.33^{a}	77.96 ± 5.68^{b}	N.D.	76.06 ± 3.48^{ab}	N.D.	**0.009**
	Leukocytes	90.81 ± 1.57	90.53 ± 1.05	91.12 ± 1.35	91.6 ± 1.22	90.57 ± 1.54	0.308
IL6 CpG2: -1096	Skeletal muscle	77.40 ± 3.14^{a}	81.01 ± 3.87^{b}	N.D.	79.04 ± 3.08^{c}	N.D.	**0.001**
	Leukocytes	90.56 ± 1.21^{a}	91.14 ± 1.16^{ab}	91.25 ± 1.09^{ab}	91.76 ± 0.81^{b}	90.67 ± 0.76^{a}	**0.029**
IL6 CpG3: -1094	Skeletal muscle	82.61 ± 3.28^{a}	84.87 ± 3.28^{b}	N.D.	83.44 ± 2.86^{a}	N.D.	**0.025**
	Leukocytes	90.96 ± 2.17	91.1 ± 2.18	91.06 ± 2.54	91.79 ± 1.86	90.76 ± 1.99	0.098
IL6 CpG4: -1069	Skeletal muscle	66.13 ± 3.74^{a}	70.78 ± 5.24^{b}	N.D.	67.31 ± 3.84^{a}	N.D.	**0.002**
	Leukocytes	88.88 ± 1.66^{a}	87.52 ± 1.90^{b}	88.28 ± 1.47^{ab}	89.23 ± 1.23^{ab}	88.36 ± 1.34^{ab}	**0.016**
IL6 CpG5: -1061	Skeletal muscle	72.41 ± 2.87^{a}	74.88 ± 3.38^{b}	N.D.	73.83 ± 3.42^{c}	N.D.	**0.001**
	Leukocytes	81.52 ± 2.98	80.91 ± 2.51	81.54 ± 1.98	81.91 ± 2.27	81.27 ± 2.82	0.166
IL6 CpG6: -1057	Skeletal muscle	74.46 ± 3.74^{a}	77.55 ± 4.23^{b}	N.D.	75.93 ± 3.86^{a}	N.D.	**0.001**
	Leukocytes	87.94 ± 2.17	88.43 ± 1.59	87.64 ± 1.43	87.91 ± 1.36	87.71 ± 2.10	0.595
IL6 CpG: Mean	Skeletal muscle	74.34 ± 3.28^{a}	77.84 ± 4.12^{b}	N.D.	75.93 ± 3.31^{a}	N.D.	**0.001**
	Leukocytes	88.44 ± 1.35	88.27 ± 1.16	88.48 ± 1.05	89.03 ± 0.89	88.22 ± 1.07	0.051

7.3.3.3 TNF

The methylation values at the individual CpG sites for the *TNF* assay are presented in Table 7.6, whereas the impact of exercise within each trial on the mean methylation across all CpG sites are presented in Figure 7.11.

Reduced Post-ex *TNF* methylation was detected, in skeletal muscle, for two CpG (CpG3: +214 & CpG4: +222) sites and the mean methylation of all CpG sites ($p > 0.05$; Table 7.6, Figure 7.11A). Non-significant trends for a main effect of time were also identified for the remaining CpG sites (CpG1: $p = 0.084$; CpG2: $p = 0.055$). A simultaneous increase of *TNF* mRNA expression was also determined Post-ex in skeletal muscle ($p = 0.027$; Figure 7.11C). No associations were determined between the mean methylation across all *TNF* CpG sites analysed and *TNF* mRNA expression ($p > 0.05$; Figure 7.12); however, Post-ex+3hr *TNF* methylation was negatively correlated with Post-ex *DNMT3a* mRNA expression ($p < 0.01$; Figure 7.12).

A delayed reduction in *TNF* DNA methylation was determined in leukocytes with reduced methylation at Post-ex+3hr for three CpG sites (CpG2-4: $p < 0.05$; non-significant trend identified for CpG1: $p = 0.057$) and the mean methylation across the whole region analysed ($p < 0.05$; Table 7.6, Figure 7.11B). Exercise did not alter leukocyte *TNF* mRNA expression ($p > 0.05$; Figure 7.11D) and no association between *TNF* methylation and mRNA expression were identified ($p > 0.05$; Figure 7.13) however, positive correlations were determined between Post-ex+1hr *DNMT3b* mRNA expression and both Post-ex and Post-ex+3hr *TNF* methylation ($p < 0.05$; Figure 7.13).

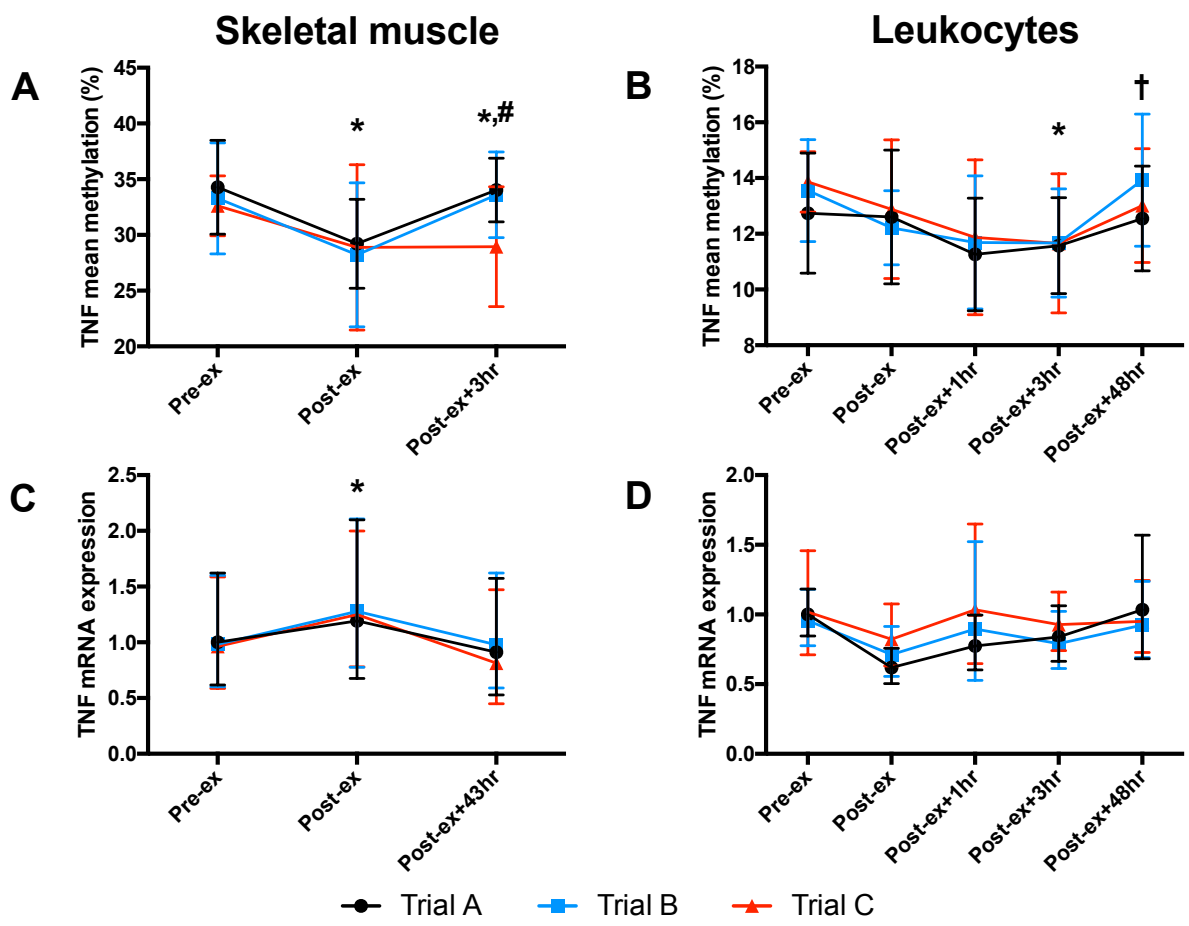

Figure 7.11 - *Impact of exercise on mean TNF DNA methylation across all CpG sites (A & B) and mRNA expression (C & D) in skeletal muscle (left-hand column) and leukocytes (right-hand column). *indicates significantly different from Pre-ex; # indicates significantly different from Post-ex; † indicates significantly different from Post-ex+3hr.*

Table 7.6 - *DNA methylation of TNF. Data presented as the mean of all trials ± standard deviation. Values not sharing a letter (a,b,c) are significantly different for simple interactions of time (p<0.05) after Bonferroni correction for multiple testing.*

CpG site	Tissue	Pre-Ex	Post-ex	Post-ex+1hr	Post-ex+3hr	Post-ex+48hr	Main effect p-value
TNF CpG1: +197	Skeletal muscle	29.63 ± 3.04	26.21 ± 3.98	N.D.	29.17 ± 3.32	N.D.	0.084
	Leukocytes	13.77 ± 1.55	13.01 ± 2.05	12.06 ± 1.81	12.17 ± 2.03	13.45 ± 1.97	0.057
TNF CpG2: +202	Skeletal muscle	24.32 ± 2.70	20.82 ± 3.40	N.D.	23.35 ± 2.83	N.D.	0.055
	Leukocytes	11.2 ± 1.61^{a}	10.86 ± 1.66^{ab}	10.04 ± 1.96^{ab}	10.01 ± 1.75^{b}	11.09 ± 1.57^{ab}	**0.048**
TNF CpG3: +214	Skeletal muscle	29.09 ± 2.59^{a}	25.37 ± 4.03^{b}	N.D.	27.87 ± 3.09^{ab}	N.D.	**0.044**
	Leukocytes	12.97 ± 1.82^{a}	12.43 ± 2.00^{ab}	11.67 ± 2.23^{ab}	11.58 ± 1.93^{b}	12.7 ± 1.99^{ab}	**0.02**
TNF CpG4: +222	Skeletal muscle	50.53 ± 3.95^{a}	42.7 ± 6.51^{b}	N.D.	48.37 ± 5.09^{ab}	N.D.	**0.012**
	Leukocytes	15.60 ± 1.76^{a}	13.97 ± 2.05^{ab}	12.6 ± 2.00^{ab}	12.78 ± 1.66^{b}	15.41 ± 2.23^{a}	**0.001**
TNF CpG: Mean	Skeletal muscle	33.39 ± 2.97^{a}	28.77 ± 4.4^{b}	N.D.	32.19 ± 3.53^{ab}	N.D.	**0.031**
	Leukocytes	13.38 ± 1.42^{a}	12.57 ± 1.71^{ab}	11.59 ± 1.77^{ab}	11.63 ± 1.65^{b}	13.16 ± 1.72^{a}	**0.004**

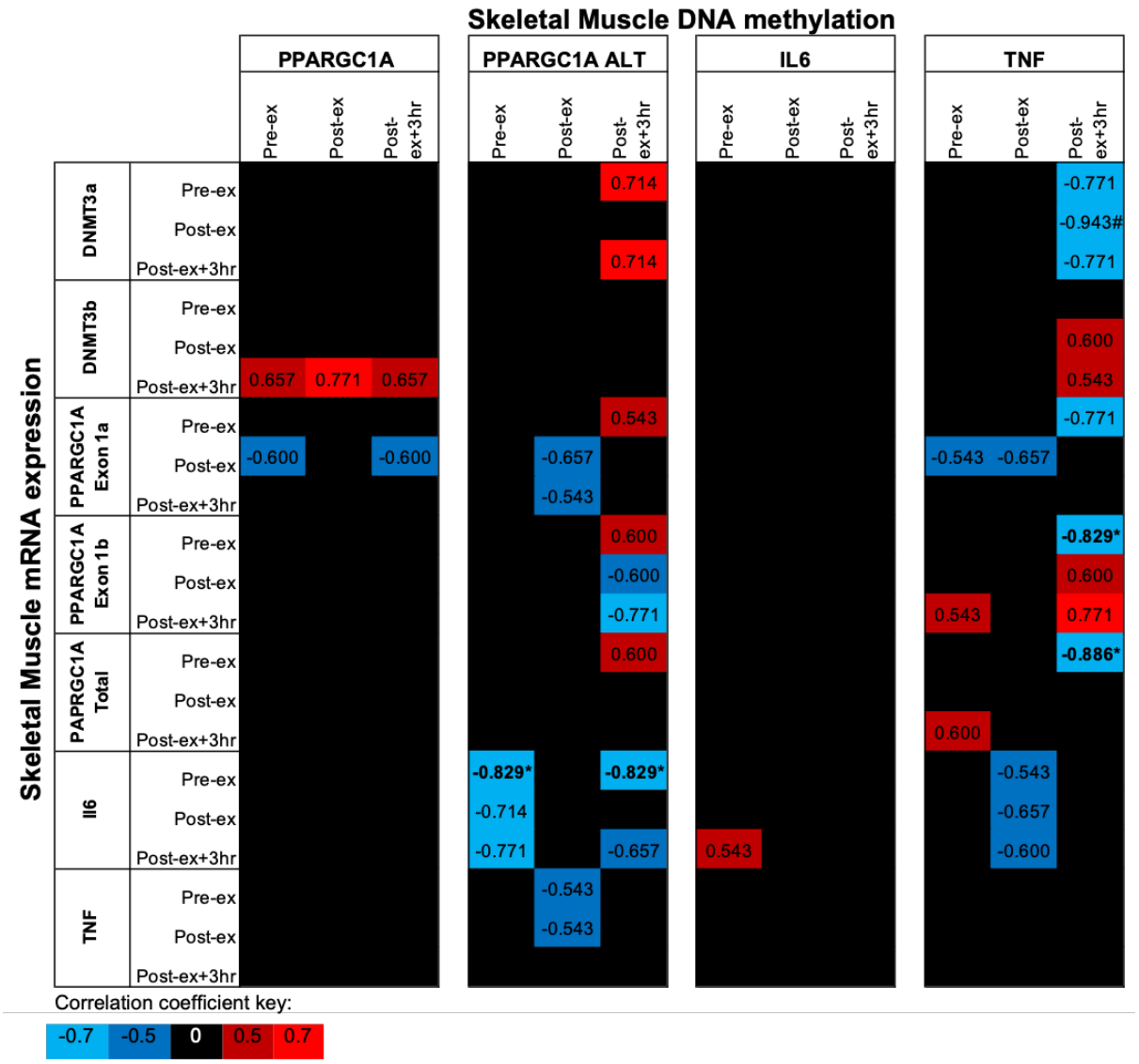

Figure 7.12 – *Spearman's Rho correlation coefficients between mean skeletal muscle DNA methylation and mRNA expression values across all trials (Trial A-C). Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. *p < 0.05, # p < 0.01.*

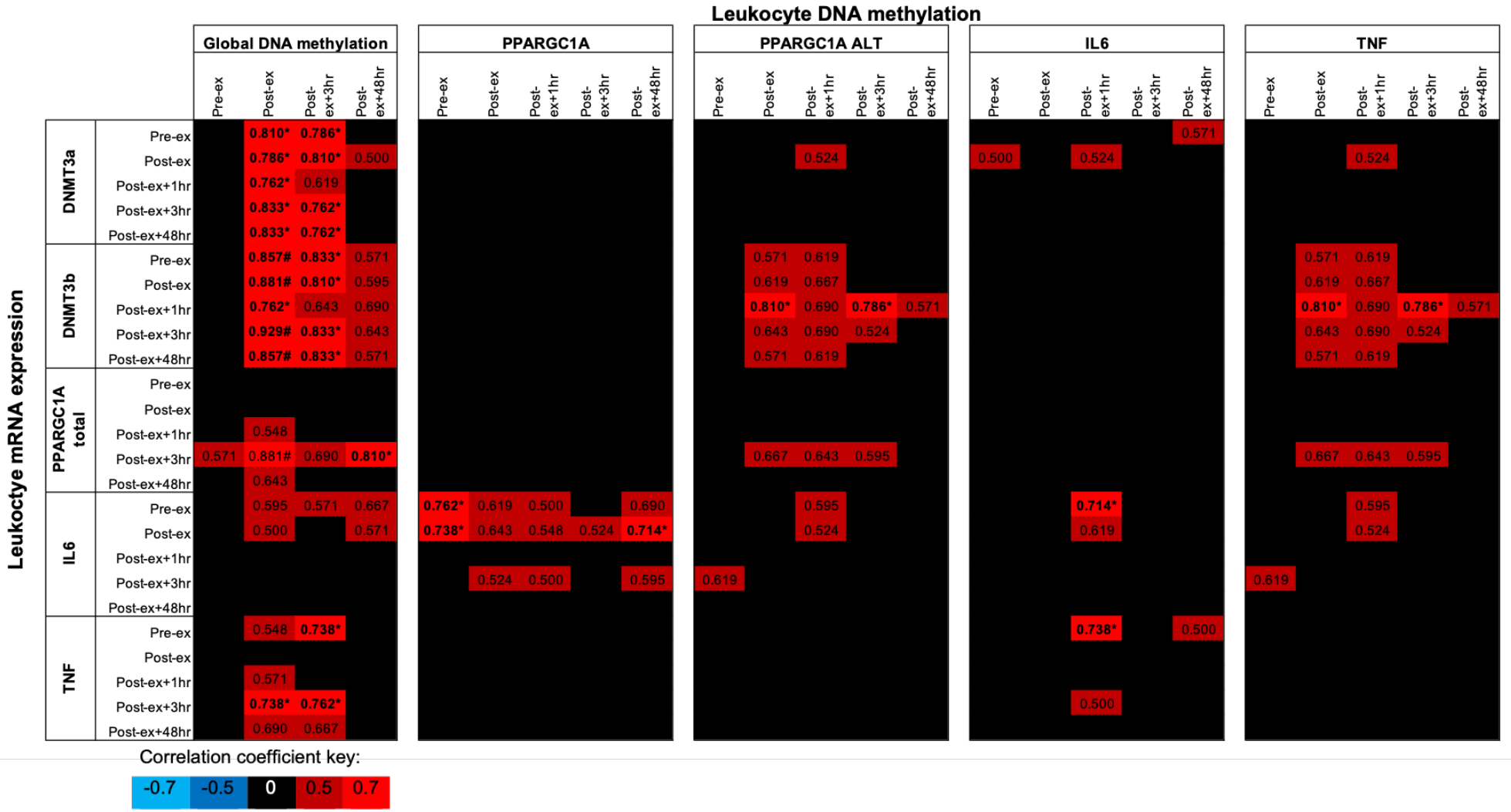

Figure 7.13 - *Spearman's Rho correlation coefficients between mean leukocyte DNA methylation and mRNA expression values across all trials (Trial A-C). Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. *p < 0.05, # p < 0.01.*

7.3.4 Associations between skeletal muscle and leukocyte DNA methylation

Significant positive correlations were identified between *PPARGC1A* DNA methylation within skeletal muscle and leukocytes ($p < 0.05$; Figure 7.14); however, no significant positive associations were identified between skeletal muscle and leukocytes for the other assays (*PPARGC1A* ALT, *IL6* and *TNF*, $p > 0.05$; Figure 7.14). Although no positive correlations were identified for the PPARGC1A ALT, IL6 or TNF assays, significant negative correlations between skeletal muscle and leukocytes were identified for each of these assays; however, the negative correlations between the tissues are not consistent across the various time points suggesting a high degree of tissue specificity for these assays (Figure 7.14).

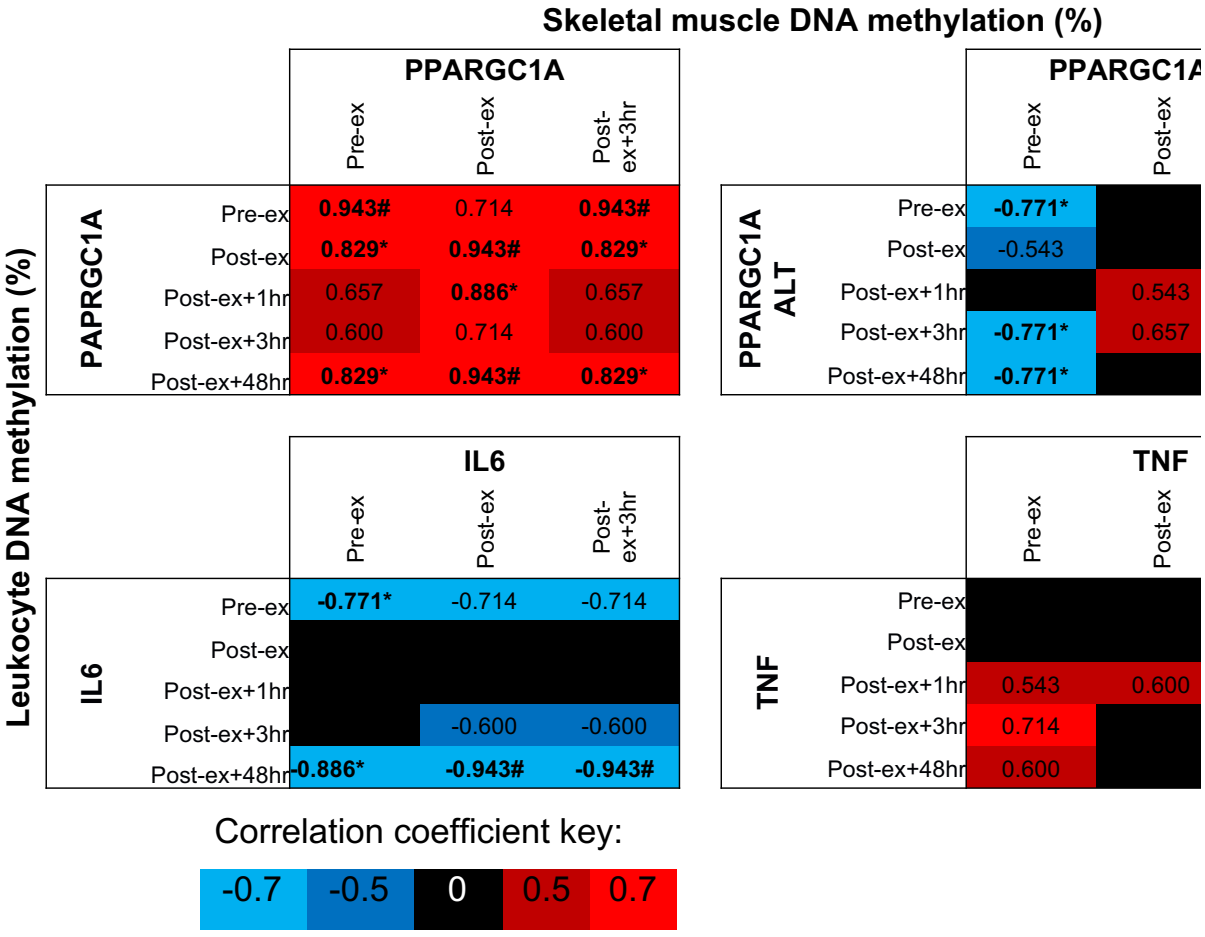

Figure 7.14 *- Spearman's Rho correlation coefficients of mean DNA methylation of all CpG sites analysed within skeletal muscle and blood leukocytes for each assay. Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. *p < 0.05, # p < 0.01.*

7.3.5 Associations between DNA methylation and post-exercise physiological markers

Figure 7.15 demonstrates the association between mean methylation across all CpG sites for each assay and physiological markers related to exercise performance, inflammation and muscle damage. A large number of significant associations have been determined and are not commented upon in detail; however, the section below describes the consistent significant associations identified.

7.3.5.1 Exercise performance

Exercise performance was not significantly associated with global, PPARGC1A or PPARGC1A DNA methylation ($p > 0.05$; Figure 7.15); however, significant correlations were identified between exercise performance and TNF DNA methylation in skeletal muscle. Strong positive correlations were identified between CMJ height and post-exercise TNF methylation ($r > 0.8$, $p < 0.05$; Figure 7.15) whereas, significant negative correlations were identified between the TNF methylation post-ex+3hr and force produced during isometric, eccentric and concentric contractions of the knee extensors ($r < -0.8$, $p < 0.05$; Figure 7.15). Within leukocytes, positive associations were determined between post-ex IL6 DNA methylation and knee extensor force production during the performance tests; however, this association only reached statistical significance for isometric force production post-ex+48hr ($r = 0.881$, $p < 0.01$; Figure 7.15).

7.3.5.2 Inflammatory cytokines

No significant associations were identified between circulating IL-6 concentrations and *IL6* DNA methylation in either blood leukocytes or skeletal muscle ($p > 0.05$). Within skeletal muscle a significant positive correlation was identified between IL-6 concentrations and *PPARGC1A* ALT methylation Post-ex+3hr ($r = 0.829$, $p < 0.05$; Figure 7.15); whereas, circulating IL-6 concentrations Post-ex+3hr were negatively associated with the Post-ex+48hr DNA methylation of *PPARGC1A* and *TNF* in leukocytes ($r = -0.714$, $p < 0.05$; Figure 7.15).

Significant negative correlations were identified between Post-ex TNF-α and skeletal muscle *PPATGC1A*, *PPARGC1A* ALT and *IL6* ($r < -0.8$, $p < 0.05$; Figure 7.15); however, no significant associations were identified with skeletal muscle *TNF* methylation or the leukocyte DNA methylation for any of the assays ($p > 0.05$; Figure 7.15).

7.3.5.3 Muscle damage markers

Neither global nor *IL6* methylation were significantly associated with markers of muscle damage ($p > 0.05$), whereas, significant correlations were identified between markers of muscle damage and DNA methylation of *PPARGC1A, PPARGC1A* ALT and *TNF* ($p < 0.05$). Within leukocytes, negative correlations were identified for between the DNA methylation of these assays and the circulating concentrations of myoglobin and CK ($r < -0.714$, $p < 0.05$; Figure 7.15); whereas, within skeletal muscle only DNA methylation of *PPARGC1A* ALT was associated with muscle damage markers ($r = 0.886$, $p < 0.05$; Figure 7.15).

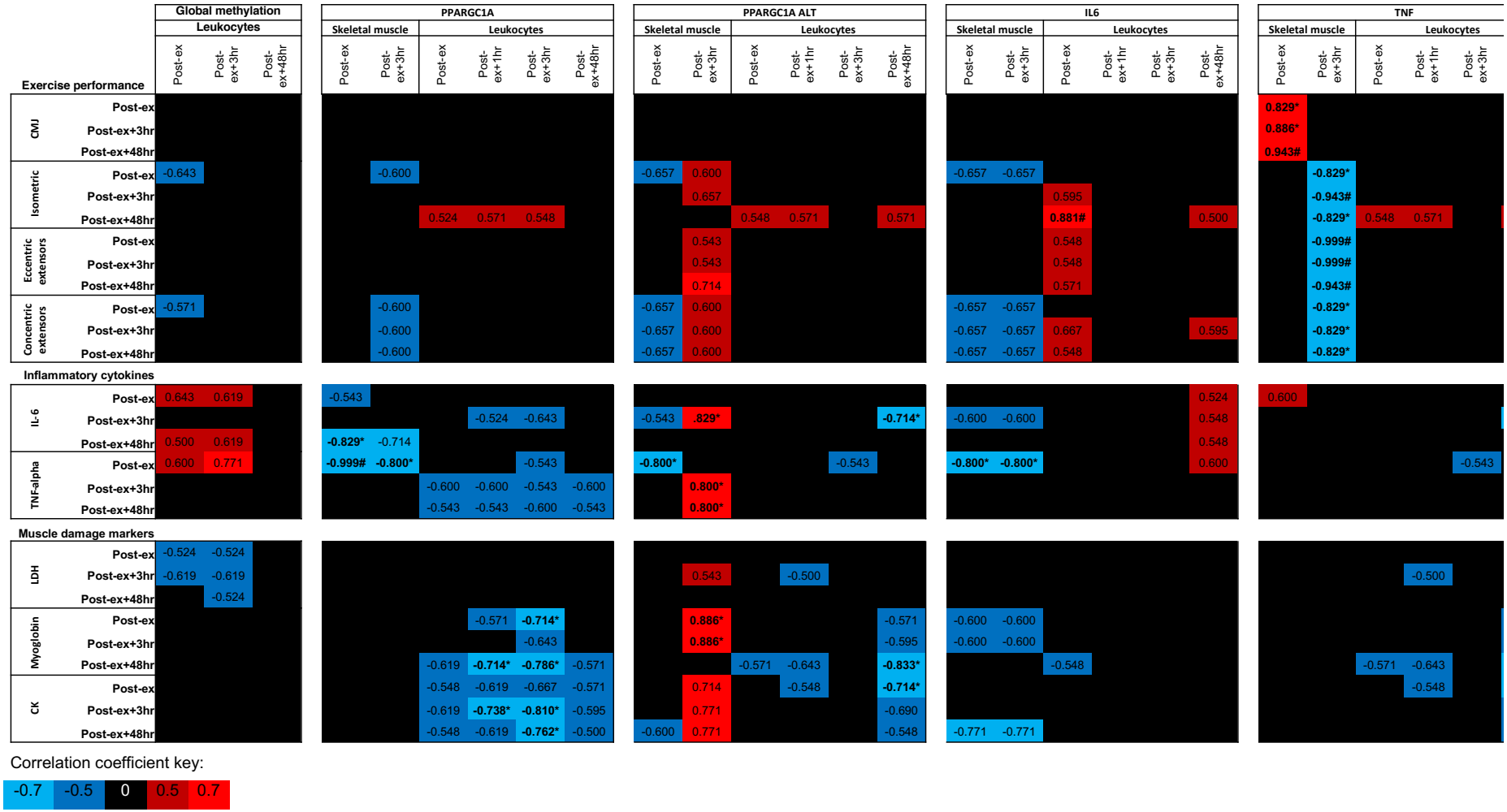

Figure 7.15 - Spearman's Rho correlation coefficients between post-exercise DNA methylation and physiological markers related to exercise performance, inflammation and muscle damage. The mean of all CpG sites assessed for each assay has been used to provide an overall view of the region of interest. Blue indicates a negative correlation; red indicates a positive correlation and black indicates correlation coefficients between -0.5 and 0.5. *p < 0.05, # p < 0.01.

7.4 Discussion

This chapter aimed to investigate the impact of acute resistance exercise on DNA methylation in leukocytes and skeletal muscle from individuals unaccustomed to resistance exercise. Further, we sought to determine whether FA supplementation or resistance training could modulate the acute response. Acute resistance exercise did not alter global DNA methylation in leukocytes; however, modulated gene-specific DNA methylation (*PPARGC1A*, *IL6* and *TNF*) was detected in both skeletal muscle and leukocytes. Acute resistance exercise also reduces the mRNA expression of *DNMT3a* and *DNMT3b* in both tissues. Neither the supplementation of FAs or eight-weeks of resistance training were sufficient to alter the impact of acute resistance exercise on DNA methylation. Significant correlations were determined between Post-ex *PPARGC1A*, *IL6* and *TNF* DNA methylation; and physiological markers related to exercise performance, inflammation and markers of muscle damage indicating potential functional consequences of the alterations to DNA methylation.

The lack of global hypomethylation in leukocytes following an acute bout of resistance exercise in the present study was an unexpected finding based on a previous report of genome-wide remodelling of the methylome with a preference towards hypomethylation (~10,000 sites decreases compared to ~7,500 sites increasing methylation) in skeletal muscle following acute resistance exercise (Seaborne et al. 2018). The lack of hypomethylation in the present study could be explained if the resistance exercise was insufficient to alter DNA methylation; however, a decrease in performance and an increase in markers of muscle damage and inflammatory cytokines were detected as a result of the resistance exercise suggesting the exercise was sufficient to alter inflammatory pathways and induce muscle damage.

A tissue-specific response between skeletal muscle and leukocytes could explain the surprising findings in the current study. A tissue-specific response to resistance training between leukocytes and skeletal muscle is suggested in the literature. The same number of CpG sites in leukocytes become hypomethylated as hypermethylated (~28,000) following resistance exercise (Denham et al. 2016); whereas, resistance training causes an increased number of sites to undergo hypomethylation in skeletal muscle (Rowlands et al. 2014; Seaborne et al. 2018). The tissue-specific response of

methylation following exercise is supported by the alteration of methylation in different molecular pathways in skeletal muscle (Nitert et al. 2012) and adipose tissue (Rönn et al. 2013) from the same individuals following a six-month aerobic training intervention.

The lack of assessment of skeletal muscle global methylation in the present study, because of a small yield of skeletal muscle from the biopsy procedure, prevents the conclusion of a tissue-specific global methylation response to acute resistance exercise. Despite no measure of global DNA methylation in skeletal muscle within the present study, the mRNA expression of the DNMT enzymes was assessed in both skeletal muscle and leukocytes. In skeletal muscle, decreased mRNA expression of *DNMT3a* and *DNMT3b* was identified following exercise; whereas, only *DNMT3b* was reduced in leukocytes. Global leukocyte methylation was positively correlated to the mRNA expression of *DNMT3a* and *DNMT3b* suggesting the expression of these enzymes may be critical in determining methylation. Indeed, altered mRNA expression has previously been associated with modulation of DNA methylation (Jaiswal et al. 2015; Kobayashi et al. 2011); therefore, hypomethylation in skeletal muscle may occur as a result of a tissue-specific modulation of DNMT enzymes. In support of this theory, Denham et al., (2016) identified reduced mRNA expression of *DNMT3b* but not *DNMT3a* following resistance training. These results suggest the lack of leukocyte global hypomethylation induced by resistance exercise in the present study may be explained by a similar number of CpG sites increasing and decreasing in methylation.

For the first time, we have investigated the impact of resistance exercise on the methylation and mRNA expression of both promoter regions of *PPARGC1A*. Acute aerobic exercise studies have demonstrated an exercise-induced hypomethylation of a single CpG site -260 upstream of the canonical *PPARGC1A* promoter and a concurrent increase in *PPARGC1A* mRNA expression (Bajpeyi et al. 2017; Barrès et al. 2012; Nitert et al. 2012); however a lack of literature exists on the impact of resistance exercise on the methylation of *PPARGC1A*. In contrast to the response of acute aerobic exercise, we identified hypermethylation of the *PPARGC1A* canonical promoter (CpG -260) in skeletal muscle following resistance exercise. Despite strong positive correlations between PPARGC1A methylation in skeletal muscle and leukocytes, we failed to detect any change in DNA methylation in leukocytes post-

exercise following the correction for multiple testing. The hypermethylation determined in the present study indicates that exercise induces mode specific changes to *PPARGC1A*. Gene expression studies have previously reported an exercise mode-specific expression pattern of the *PPARGC1A* isoforms from the specific promoters (Ruas et al. 2012; Silvennoinen et al. 2015), suggesting a potential epigenetic role in the transcriptional control of these isoforms.

Based on the typical relationship between methylation and mRNA expression we expected the *PPARGC1A* ALT to become hypomethylated following exercise as a mechanism to increase the mRNA expression of Exon 1b derived *PPARGC1A* reported in previous studies (Ruas et al. 2012; Silvennoinen et al. 2015). In the present study, we identified significant hypermethylation of *PPARGC1A* ALT in both skeletal muscle and leukocytes following acute resistance exercise. Despite hypermethylation of the *PPARGC1A* alternative promoter, we identified a substantial increase in Exon 1b derived mRNA expression in skeletal muscle. The relationship identified between promoter methylation and mRNA expression of *PPARGC1A* ALT is the opposite to the normal reported relationship; however, a recent study examining DNA methylation in tumour samples indicate that ~30% of CpG sites downstream of the TSS are positively associated with mRNA expression (Spainhour et al. 2019). There is a sparsity of literature investigating the impact of exercise on the methylation status of *PPARGC1A* ALT. The only previous study to investigate the impact of exercise on the methylation of *PPARGC1A* ALT failed to determine any modulation of methylation at either of the *PPARGC1A* promoters in mice one-hour following an acute bout of aerobic exercise (Lochmann et al. 2015). It is difficult to compare the results of the present study with this report because of key methodological differences including the species, exercise-mode and timepoint following exercise.

Positive associations were identified between muscle damage markers and *PPARGC1A* ALT methylation post-exercise in skeletal muscle, suggesting that exercise-induced muscle damage may be required to alter the methylation of *PPARGC1A* ALT. The muscle damage response in the study by Lochmann et al., (2015) is not reported; however, eccentric exercise is demonstrated to induce greatest muscle damage which potentially explains the lack of association between aerobic exercise and *PPARGC1A* ALT methylation in the previous study. Negative correlations

were also identified between both *PPARGC1A* promoters and the post-exercise circulating concentrations of TNF-α. These data support the previous associations between increased *PPARGC1A* mRNA expression and a reduction in the systemic levels of inflammatory cytokines (Handschin and Spiegelman 2008). Future work is required to confirm the relationship between methylation of these CpG sites within *PPARGC1A* ALT, the isoform-specific expression of *PPARGC1A* and the physiological consequence of these relationships.

For the first time, acute exercise is demonstrated to be sufficient to induce a tissue-specific *IL6* DNA methylation response. Immediately post an acute bout of resistance exercise the methylation of all *IL6* CpG sites analysed displayed significant hypermethylation, and by Post-ex+3hr the methylation values had decreased or returned to Pre-exercise level. Increased mRNA expression of *IL6* was also detected Post-ex, as previously mentioned the opposite relationship would be expected between methylation and mRNA expression; however, in line with the results of the current study a positive association has previously been reported between a single CpG site (-666) closer to the TSS of the *IL6* promoter and *IL6* mRNA expression (Ma et al. 2016). The impact of resistance exercise on leukocyte *IL6* methylation is not as clear. There was no impact of exercise on the mean methylation of all CpG sites; however, a single CpG site (CpG4: -1069) decreased in methylation Post-ex, whereas, Post-ex+3hr a different CpG increased in methylation (CpG2: -1096). The small changes in leukocyte methylation were insufficient to alter *IL6* mRNA expression in the present study. The tissue-specific modulation of *IL6* methylation and subsequently mRNA expression in the present study suggests that altered DNA methylation may be responsible for the increased production of IL-6 in exercising skeletal muscle (Pedersen and Febbraio 2008; Steensberg et al. 2000). It has been suggested the increased skeletal muscle production of *IL6* may induce an anti-inflammatory response by increasing the expression of IL-1ra and IL-10 (Steensberg et al. 2003) and inhibiting TNF-α production (Gleeson et al. 2011; Petersen and Pedersen 2006). In support of the anti-inflammatory role of muscle produced IL-6, we identified a negative correlation between skeletal muscle *IL6* methylation and circulating concentrations of TNF-α.

While the acute bout of exercise in the present study hypermethylated *PPARGC1A* and *IL6*, hypomethylation of the first exon of *TNF* was detected in both skeletal muscle

and leukocytes. Alongside the hypomethylation of *TNF*, we detected an increase of *TNF* mRNA expression in skeletal muscle and correlations with exercise performance; whereas, no change in *TNF* mRNA expression or associations with exercise performance were identified in leukocytes. Previously, *TNF* has been reported to be hypermethylated in leukocytes from elderly individuals who maintained or increased their energy expenditure by 500 kcal/wk over an eight-year period (Shaw et al. 2014). These data suggest differential regulation of *TNF* by acute resistance exercise and long-term physical activity. Potentially the acute decrease in *TNF* methylation and increase in mRNA expression following resistance exercise could be involved in the adaptive response to muscle-damaging exercise via activation of satellite cells and increased expression of the myogenic differentiation factors MyoD and Myogenin (Chen et al. 2007; Li 2013).

Conversely, the increase in *TNF* methylation following long-term physical activity may function to reduce the systemic levels of inflammation associated with disease states and skeletal muscle atrophy (Sharples et al. 2010). In support of these results, administration of TNF-α to muscle cells reduces the regenerative capacity (Sharples et al. 2016). The exposure of mouse C2C12 cell to an early dose of TNF-α has also been demonstrated to alter MyoD promoter hypermethylation and reduced differentiation and increased atrophy when cells are exposed to a later dose of TNF-α, compared to cells which only encounter a later dose of TNF-α. In support of a role of *TNF* methylation in the hypertrophic response, we identified significant correlations between *TNF* methylation and isometric, concentric and eccentric force produced by the knee extensors. These data provide strong evidence for an epigenetic role for *TNF* in controlling skeletal muscle mass which is regulated by stimulus provided by acute and chronic exercise.

While acute resistance exercise altered DNA methylation patterns in the present study, we did not identify any impact of exercise training or FA supplementation on DNA methylation. Reductions in systemic levels of inflammation have been reported following exercise training (Beavers et al. 2010; Flynn et al. 2007; Gleeson et al. 2011) and FA supplementation (Calder 2015; Rosignoli et al. 2013; Yarla, Polito, and Peluso 2017). Including the methylation of a single CpG site further downstream of the *IL6* gene associated with the n-3 PUFA content in blood (Ma et al. 2016) and

administration of EPA was sufficient to dampen the impact of *TNF* administration on MyoD mRNA expression (Saini et al. 2017). These data suggest the CpG sites investigated in the present study may regulate the acute local inflammatory response and not the chronic systemic inflammatory response. Alternatively, because the participants were young and healthy, there may not have been a systemic inflammatory response to be resolved. Repetition of the present study in a cohort of older adults with chronic inflammation or within an inflammatory disease population will allow the determination if the selected CpG sites are only involved in the acute response or whether the lack of association is due to the selection of young and healthy participants.

The time course of the acute resistance exercise-induced changes in DNA methylation has not previously been reported. The only previous study to investigate the impact of acute resistance exercise on DNA methylation quantified methylation pre- and 30 min post-exercise (Seaborne et al. 2018). The inclusion of a further time point in the present study (Post-ex+3hr) allows the determination that the methylation response to acute resistance exercise is a transient event, similar to the previously reported association following aerobic exercise (Barrès et al. 2012). Interestingly, there is a lack of literature on exercise-induced changes to DNA methylation in leukocytes. The only previous report of acute exercise in leukocytes failed to identify any significant CpG sites immediately post or 24-hours post-exercise (Robson-Ansley et al. 2014). In the present study, altered DNA methylation only occurred immediately following exercise for one CpG site in *IL6*, the majority of CpG sites displayed a delayed response and altered DNA methylation at Post-ex+3hr. These data suggest the lack of modulated DNA methylation detected in the study by Robson-Ansley and colleagues (2014) may be explained the selection of sampling time points. Future work is required to accurately determine the time course of methylation changes following acute exercise.

An important consideration for the skeletal muscle DNA methylation detected in the present study, and any studies investigating the impact of muscle-damaging exercise on DNA methylation within skeletal muscle, is the infiltration of leukocytes into skeletal muscle following muscle damage and the potential impact it may have on DNA methylation profiles. There is contrasting evidence of the time course of leukocyte infiltration, while some studies report no infiltration during the initial 3 hours post-

exercise (the time course of the present study) (MacIntyre et al. 1996; Mahoney et al. 2008; Raastad et al. 2015), others have reported leukocyte infiltration into skeletal as soon as 30 min post-exercise (Paulsen et al. 2010). The infiltration of leukocytes into skeletal muscle following exercise will lead to the inclusion of leukocyte genetic material into the Post-ex DNA samples which could impact the determination of DNA methylation. In the present study, significant differences in the methylation profile of leukocytes and skeletal muscle for each gene was detected at baseline (Trial A, Pre-ex; Table 7.3); therefore, if leukocyte infiltration has occurred, it could be the causal factor for the change in DNA methylation following exercise in the present study. Future studies should consider the potential impact of leukocyte infiltration and assess the expression of markers unique to leukocytes to confirm the absence of their contribution to the genetic material used for analysis.

7.5 Conclusion

In conclusion, acute resistance exercise was sufficient to alter DNA methylation of *PPARGC1A*, *IL6* and *TNF* in both skeletal muscle and leukocytes; however, differences in the direction of the methylation response between these two tissues suggests a tissue-specific response. A tissue-specific response is further demonstrated by the lack of positive correlations, with the exception of *PPARGC1A*, between the DNA methylation within skeletal muscle and leukocytes. The tissue-specific response between skeletal muscle and leukocytes is an important finding because leukocyte methylation is commonly used as a surrogate for other tissues. Neither exercise training or FA supplementation was sufficient to alter either gene-specific or global DNA methylation. The lack of alteration of DNA methylation as a result of exercise training for any of the genes analysed in the present study suggests that these methylation changes occur independently of training status; however, this may be due to the selection of a young cohort of healthy males with a lack of chronic inflammation. Repetition of this study in a cohort of individuals suffering from inflammatory diseases would allow the determination if the lack of association of training and supplementation is because of the selection of healthy participants in the current study.

Chapter 8 General discussion

8.1 Overview and thesis aims

A wealth of literature has researched the impact of genetic polymorphisms, mainly SNPs, with exercise and health; whereas, the impact of exercise on epigenetic modifications, which possess a critical role in transcriptional control and may be the key to understanding the adaption process and potential health benefits to exercise and FA supplementation, are only beginning to be elucidated. This thesis aimed to:

1. Identify the impact of different acute exercise stimuli (aerobic and resistance) on global and gene-specific methylation.

2. Investigate whether the impact of acute exercise can further be modulated by the supplementation of dietary fatty acids.

3. Determine whether the expression of DNMT enzymes are associated with the modulation of DNA methylation in an attempt to identify a potential underlying mechanism.

4. Establish whether modulated DNA methylation as a result of the previously mentioned interventions is associated with physiological markers.

8.2 Summary of the research

The use of different exercise bouts in the different chapters of this thesis allowed the comparison of the impact of different exercise stimuli on DNA methylation. While an acute bout of exercise to volitional fatigue was insufficient to alter DNA methylation (**Chapter 5**), significant alterations in methylation were detected following a one-hour cycling bout (Global and *PPARGC1A*; **Chapter 6**), and an acute bout of resistance exercise (*PPARGC1A*, *IL6* and *TNF* ; **Chapter 7**). A period of eight weeks of resistance exercise did not alter global or gene-specific DNA methylation and the response to an acute bout of exercise following the training period was not different to the response before training (**Chapter 7**).

FA supplementation alone did not alter either global or gene-specific DNA methylation in the present thesis (**Chapters 5 - 7**). In **Chapter 5**, an interaction between n-3 PUFA supplementation and a modulated DNA methylation response following exercise was identified for a single *IL6* CpG site. Interestingly following supplementation, the n-3 PUFAs content of whole blood associated with methylation of the same *IL6* CpG site suggesting the modulation of fatty acid content may alter DNA methylation following an exercise stimulus. The exercise stimulus in **Chapters 6 and 7** was not further modulated by FA supplementation.

All three exercise stimuli were sufficient to reduce *DNMT3a* and 3b mRNA expression (**Chapters 5 - 7**); however, *DNMT1* mRNA expression was only reduced following an acute bout of exercise to volitional fatigue (**Chapter 5**). The reduction in all three DNMTs following exercise in **Chapter 5**, without a change in global or gene-specific DNA methylation (*PPARGC1A* and *IL6*), questions the functional consequence of altered DNMT mRNA expression. Differential mRNA expression of *DNMT1* was detected in following n-3 PUFAs and EVOO supplementation in **Chapter 6**; however, FA supplementation did not associate with DNMT expression in **Chapter 5 or 7**. While mRNA expression has previously been associated with global methylation levels (Jaiswal et al. 2015; Kobayashi et al. 2011), potentially DNMT enzyme activity would provide a better measure to investigate associations with DNA methylation.

8.3 Global DNA methylation

Global DNA methylation was determined in blood leukocytes in all three experimental chapters (**Chapters 5 - 7**) in the current thesis. Previously significant hypomethylation of the genome is reported in skeletal muscle following an acute bout of exercise (Barrès et al. 2012; Seaborne et al. 2018) and exercise training interventions (Nitert et al. 2012; Rowlands et al. 2014; Seaborne et al. 2018), whereas in leukocytes the results are mixed with some studies reporting no change in global methylation (Denham et al. 2016; King-Himmelreich et al. 2016; Robson-Ansley et al. 2014) and others report significant hypomethylation (Denham, O'Brien, Marques, et al. 2015; Dimauro et al. 2016) following exercise. The results obtained within the present thesis match the lack of consistency in the literature surrounding the impact of different exercise interventions on global methylation with one study indicating reduced global

methylation following acute exercise (**Chapter 6**); whereas the other two studies (**Chapters 5 and 7**) failed to identify an association between exercise and global DNA methylation. The use of different bouts of exercise (altered mode, intensity and duration) induced different physiological demands and therefore are likely to regulate different molecular pathways potentially explaining the lack of clarity in overall impact on global DNA methylation because of different CpG sites undergoing hyper- and hypomethylation.

The supplementation of FAs did not alter the global DNA methylation in the present thesis (**Chapters 5 – 7**). The literature surrounding the global impact of FA supplementation is unclear, with a study reporting decreased LINE-1 methylation following n-3 PUFA supplementation in Alzheimer's patients (Karimi et al. 2017), whereas, the supplementation of n-3 PUFAs in overweight and obese adults altered the DNA methylation at 306 CpG sites with 93% of them displaying hypermethylation (Tremblay et al. 2017). Potentially the use of diseased populations in the previous literature explains the inconsistencies detected. *LINE-1* methylation is reported to be increased in Alzheimer's patients compared to healthy controls (Di Francesco et al. 2015); therefore, the supplementation of n-3 PUFA in these Alzheimer's patients may act to restore global DNA methylation to the normal level detected in healthy individuals explaining the lack of association the present thesis which used healthy individuals.

8.4 Gene-specific methylation

The exercise stimulus in **Chapter 5** was insufficient to alter the DNA methylation of *PPARGC1A* in leukocytes; whereas the acute bouts of aerobic exercise in **Chapter 6** decreased the methylation of *PPARGC1A*. These results are in agreement with the impact of aerobic exercise in skeletal muscle (Barrès et al. 2012; Nitert et al. 2012). In both **Chapter 5 and Chapter 6** *PPARGC1A* methylation was positively correlated with measures of exercise performance, suggesting the methylation response to exercise may be dependent on an individual's aerobic fitness, indicating a potential molecular mechanism for exercise-induced adaptation. When the methylation status of *PPARGC1A* was investigated following resistance exercise (**Chapter 7**), we identified significant hypermethylation of the -260 CpG site. These results suggest that the

regulation of methylation of the same CpG site can be influenced by exercise mode. It was expected that the increased methylation of the canonical promoter would result in hypomethylation of the alternative promoter as a mechanism controlling the transcription of alternative isoforms of *PPARGC1A* which have been reported in gene expression studies (Ruas et al. 2012; Silvennoinen et al. 2015). Despite finding the expected increased mRNA expression, hypermethylation of the *PPARGC1A* ALT was identified; however, unlike Chapters 5 and 6 no correlation was identified between *PPARGC1A* DNA methylation and exercise performance. To our knowledge, no previous literature exists on investigating the impact of these CpG sites on mRNA expression.

Aerobic exercise alone did not alter the methylation of *IL6* in the present thesis (**Chapters 5 and 6**); however, we did identify a significant impact of resistance exercise (**Chapter 7**). A complicated relationship was identified in leukocytes with one CpG site (-1096) increasing and another CpG site (-1069) decreasing in methylation; however, these changes in methylation did not result in altered mRNA expression. Conversely, in skeletal muscle, all six CpG sites analysed increased in methylation following resistance exercise; with a simultaneous increase in mRNA expression. The minimal impact of exercise on *IL6* DNA methylation in leukocytes regardless of exercise mode supports the tissue-specific methylation response detected in **Chapter 7**. Although there is a lack of other studies investigating the impact of exercise on *IL6* DNA methylation, the gene expression of *IL6* has been reported to increase in skeletal muscle but not leukocytes following acute exercise (Gjevestad et al. 2017) which is in agreement with the results detected in this thesis.

We investigated the impact of aerobic (**Chapter 6**) and resistance exercise (**Chapter 7**) on the DNA methylation of 4 CpG sites in the first exon of *TNF*. In both chapters we detected significant correlations between *TNF* methylation and exercise performance; however, only acute resistance exercise (**Chapter 7**) was sufficient to reduce the methylation of these CpG sites in both skeletal muscle and leukocytes. The only previous association with *TNF* methylation with exercise demonstrated that maintenance or increasing long-term (eight-year period) physical activity in elderly participants is sufficient to increase leukocyte methylation of *TNF*. It is likely that the differences in the reported impact of exercise on *TNF* methylation are caused by the

difference between acute local and chronic systemic TNF-α expression. We speculate that following acute resistance exercise *TNF* is hypomethylation as a mechanism to increase the local concentration in skeletal as part of the adaptive response to muscle damage; whereas, the hypermethylation of *TNF* following long-term physical activity likely functions as a mechanism to reduce the systemic levels of inflammation which are associated with adverse health outcomes. Data from the present thesis (**Chapter 6**) and a previous report by Hermsdorff et al., (2013) identify negative relationships between *TNF* methylation and measures of adiposity. Taken together the long-term benefits of regular exercise, such as reduced adiposity, may subsequently increase *TNF* DNA methylation levels and as a result, reduce *TNF* mRNA expression and the chronic low-grade inflammation levels associated with increased adiposity; whereas, the hypomethylation and subsequent increased mRNA expression of *TNF* following resistance exercise may stimulate skeletal muscle repair.

8.5 DNMT mRNA expression

The mRNA expression of the DNMT enzymes was investigated in each experimental chapter in an attempt to investigate potential mechanisms controlling the DNA methylation response. In each experiment chapter, we identified a reduction in DNMT mRNA expression as a result of exercise, and in **Chapter 6** the supplementation of FAs was sufficient to alter *DNMT1* mRNA expression. While exercise and FA supplementation may directly influence *DNMT* expression, these interventions may modulate *DNMT* expression by intermediary mechanisms. The expression of several miRNAs, including miRNA-29 -130 and -148, are associated with: DNMT expression (Duursma et al. 2008; Fabbri et al. 2007; Garzon et al. 2009; Xu et al. 2017), exercise (Silva et al. 2017) and FA supplementation (Chakraborty et al. 2017; D'Amore et al. 2016; Roessler et al. 2017). IL-6 protein levels have been reported to regulate *DNMT* expression (Foran et al. 2010; Hodge et al. 2001; Horsburgh et al. 2015) via the modulation of miRNA (Braconi et al. 2010). Future work should investigate the impact of these interventions on the activity of DNMTs and TETs to determine whether modulation of these critical enzymes following exercise and FA supplementation is sufficient to alter DNA methylation.

8.6 Selection of tissue for analysis

While the DNA sequence is identical across all cells within an individual, the same is not true regarding epigenetic signatures, each tissue and potentially each cell contains a unique methylation profile (Roadmap Epigenomics Consortium et al. 2015). The selection of target tissue for analysis is one of the most important considerations when conducting epigenetic research. Biologically interesting tissues for epigenetic analysis following environmental stimuli are sometimes not practically or ethically feasible, which leads to the selection of an alternative tissue as a surrogate from which the methylation status within the target tissue can be postulated. Circulating blood cells are a commonly selected surrogate tissue because the collection of blood is a minimally invasive procedure and since blood cells circulate throughout the body, it comes into contact with various organ and biological systems; therefore, it is thought to be a good systemic marker of methylation profiles (Jin and Liu 2018).

The two most commonly used tissues to investigate DNA methylation following exercise are skeletal muscle and blood leukocytes; whereas, the impact of FAs on DNA methylation has almost exclusively been investigated in blood-derived cells. In each of the experimental chapters (**Chapters 4 – 7**) of this thesis, we selected to use blood leukocytes as the tissue for analysis of DNA methylation. Circulating blood comprises a group of heterogeneous cell types, which is a problem because the individual cell types contain specific methylation profiles which has the potential to confound measurement of DNA methylation (Adalsteinsson et al. 2012). Potentially a change in methylation may be detected or missed because the interventions resulted in a change in the proportion of blood cell types. Exercise has been demonstrated to alter the leukocyte cell populations (Natale et al. 2003); therefore, it is essential to account for tissue composition to ensure accurate determination of the impact of exercise on DNA methylation. A strength of the present thesis is the inclusion of blood cell counts for each sample collected which enabled a statistical correction (according to Jones et al. 2017) to be applied to the methylation value based on the quantity of leukocytes (lymphocytes, neutrophils, monocytes, basophils and eosinophils) present in the sample at the particular time point. While the cell counts obtained to allow the adjusted for the main groups of leukocytes, subgroups exist which contain distinct

methylome, for example, B and T cells (Reinius et al. 2012) which have not been accounted for in this thesis.

In **Chapter 7** we also collected skeletal muscle tissue to identify any tissue-specific responses. No previous study has investigated the differences in methylation between these two tissues. Differences between the two tissues were identified at baseline and in response to exercise highlighting the importance of carefully selecting the target tissue for analysis. Association analysis of the methylation of the assays in skeletal muscle and leukocytes indicated positive correlations existed for PPARGC1A; whereas, no correlation or negative correlations existed for *PPARGC1A* ALT, *IL6* and *TNF* suggesting a tissue specific response for these assays. Interestingly, even when the same direction of methylation change was determined in **Chapter 7**, the time course of methylation changes in response to acute exercise was different between the tissues. Decreased *TNF* methylation was determined immediately post-exercise in skeletal muscle; whereas, in leukocytes the response was delayed until three-hours post-exercise. Interestingly, the time course of this change is in line with the reported infiltration of leukocytes into skeletal (MacIntyre et al. 1996; Mahoney et al. 2008; Raastad et al. 2015). Previous work in mice has demonstrated the time course of neutrophil infiltration into skeletal mirrors the neutrophil-related chemokine signalling response (Nicholas et al. 2015). These data potentially suggest an epigenetic signal in leukocytes promotes the infiltration into skeletal muscle; however, future studies are required to elucidate the relationship.

8.7 Limitations and future directions

While interesting findings were determined in this thesis, the research described is not without its limitations. A common theme amongst intervention studies is the use of a small sample size of the cohort used for investigation. The largest sample size used in the present study was ten participants; however, this is in accordance with previous literature investigating the impact of exercise on DNA methylation patterns (Denham et al. 2016; Denham, O'Brien, Marques, et al. 2015; Robson-Ansley et al. 2014; Rowlands et al. 2014; Seaborne et al. 2018; da Silva et al. 2017). Whereas, studies investigating the impact of FA supplementation have typically used larger sample sizes to identify an impact of FA supplementation (Aslibekyan et al. 2014; Karimi et al. 2017;

Ma et al. 2016; Voisin et al. 2014). Low sample size in human research study reduces statistical power and increases the likelihood that a statistically significant finding represents a false positive result (Dumas-Mallet et al. 2017). The samples sizes used for the research within the present thesis were selected based on the previous exercise studies detected significant changes in DNA methylation, alongside, difficulty in recruiting participants and consideration of analysis costs. Potentially the lack of consistent findings of altered DNA methylation following FA supplementation is the result of underpowered research.

The selection of analysis methods for the determination of DNA methylation in the present study are not without limitations. Firstly, while LUMA is a commonly used surrogate for global DNA methylation, on correlation was identified between LUMA and the gold-standard method for assessing global methylation HPLC (Lisanti et al. 2013). Further an issue with all global methylation methods is that when no difference between time points is identified (**Chapter 5 and 7**), it does not mean that there is no epigenetic consequence of the intervention because the same number of CpG sites may increase and decrease in methylation resulting in a lack of difference in global methylation (Lindholm et al. 2015). The gene-specific DNA methylation analysis using pyrosequencing also has limitations. Firstly, we are limited to determining the levels of methylation in a small number of CpG sites within each of the genes of interest. In total, we assessed the DNA methylation of 14 CpG sites (only 7 CpG sites in **Chapter 5**) out of the ~28 million CpG sites in the genome (Luo, Lu, and Xie 2014). Other analysis methods (i.e. EWAS) allow the investigation of thousands of CpG sites throughout the genome and allow the identification of common molecular pathways which are affected by interventions. However, EWAS studies are not without limitations including multiple testing, the generation of artefactual data and the need for complicated normalisation (Dedeurwaerder et al. 2014). Another limitation of pyrosequencing is the inability to distinguish between 5mC and 5hmC; however, this is a flaw of all bisulfite bases assessments of DNA methylation. Discriminating between 5mC and 5hmC can be important because these two modifications have been suggested to have opposite effects chemically and biologically

The work contained within this thesis elucidated a number of interesting findings within the field of ‘exercise-epigenetics’; however, as this is a relatively recent domain a

number of fundamental questions remain to be answered before we understand the multifaceted roles epigenetic modifications have on adaptation to exercise and the modification of the diet with bioactive molecules such as FAs. For example, decreases in DNA methylation is likely to increase transcription; however, the functional consequence of the modulation of mRNA expression is going to be dependent on the underlying genetic sequence and other epigenetic modifications, including miRNA expression and histone modifications. To gain a full insight into the transcriptional control, all of these genetic and epigenetic factors should be considered. Although not included in the present thesis, mRNA expression of TET enzymes and measures of DNMT and TET activity would help to elucidate the mechanisms which induce alterations to DNA methylation patterns following lifestyle interventions. Determination of the precise mechanisms would assist in designing therapeutic and non-therapeutic to improve health in at-risk populations.

One of the most important areas which need to be investigated is the time course of changes to DNA methylation following acute exercise. In **Chapter 7** we identified a clear tissue-specific response to the time course of changes to DNA methylation in response to acute exercise; an immediate modulation of DNA methylation was detected in skeletal muscle, whereas, a delayed alteration in methylation at three-hours post-exercise was identified in leukocytes. A number of acute exercise studies have only investigated methylation pre and at a single time point post-exercise (Bajpeyi et al. 2017; Seaborne et al. 2018; da Silva et al. 2017); whereas, inconsistent time points have been used in other studies (Barrès et al. 2012; Lane et al. 2015; Robson-Ansley et al. 2014). Differences in results between studies may be explained by the selection of different time points for determination of methylation.

The present research used healthy, disease-free, physically active participants and determined modulated DNA methylation following acute exercise. It would be interesting to determine what impact these interventions had in phenotypically diverse individuals; such as, sedentary individuals, inflammatory disease patients and individuals with chronically elevated systemic levels of inflammation.

8.8 Final conclusions

This thesis identified novel findings for the role of exercise interventions and FA supplementation on DNA methylation and mRNA expression. We identified that the different exercise interventions utilised in this thesis altered DNA methylation differently. Despite the differences in methylation we identified a consistent decrease in the mRNA expression which we suggest may be a mechanism to alter DNA methylation following exercise; however future mechanistic studies are required to confirm these associations. There was a limited impact of FA supplementation on DNA methylation patterns despite finding that *IL6* methylation was positively associated with whole blood n-3 PUFA content following supplementation. Potentially the use of a small number of participants who were healthy and physically active may explain the lack of association with FA supplementation. Future work should replicate the research in a larger cohort of individuals who suffer from inflammatory disease or chronic systemic inflammation to identify whether FA supplementation may modulate DNA methylation to reduce levels of inflammation in these participants.

References

Adalsteinsson, Bjorn T., Haukur Gudnason, Thor Aspelund, Tamara B. Harris, Lenore J. Launer, Gudny Eiriksdottir, Albert V. Smith, and Vilmundur Gudnason. 2012. “Heterogeneity in White Blood Cells Has Potential to Confound DNA Methylation Measurements.” *PLoS ONE* 7(10):1–9.

Alaskhar Alhamwe, Bilal, Razi Khalaila, Johanna Wolf, Verena Bülow, Hani Harb, Fahd Alhamdan, Charles S. Hii, Susan L. Prescott, Antonio Ferrante, Harald Renz, Holger Garn, and Daniel P. Potaczek. 2018. “Histone Modifications and Their Role in Epigenetics of Atopy and Allergic Diseases.” *Allergy, Asthma and Clinical Immunology* 14(1).

Alegría-Torres, Jorge Alejandro, Andrea Baccarelli, and Valentina Bollati. 2011. “Epigenetics and Lifestyle.” *Epigenomics* 3(3):267–77.

Alibegovic, a C., M. P. Sonne, L. Højbjerre, J. Bork-Jensen, S. Jacobsen, E. Nilsson, K. Faerch, N. Hiscock, B. Mortensen, M. Friedrichsen, B. Stallknecht, F. Dela, and A. Vaag. 2010. “Insulin Resistance Induced by Physical Inactivity Is Associated with Multiple Transcriptional Changes in Skeletal Muscle in Young Men.” *American Journal of Physiology. Endocrinology and Metabolism* 299(5):E752-63.

Allfrey, V. G., R. Faulkner, and A. E. Mirsky. 1964. “Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis.” *Proceedings of the National Academy of Sciences of the United States of America* 51(1938):786–94.

Amaral, Cátia Lira Do, Fermín I. Milagro, Rui Curi, and J. Alfredo Martínez. 2014. “DNA Methylation Pattern in Overweight Women under an Energy-Restricted Diet Supplemented with Fish Oil.” *BioMed Research International* 2014.

Amarasekera, Manori, Paul Noakes, Deborah Strickland, Richard Saffery, David J. Martino, and Susan L. Prescott. 2014. “Epigenome-Wide Analysis of Neonatal CD4+ T-Cell DNA Methylation Sites Potentially Affected by Maternal Fish Oil Supplementation.” *Epigenetics* 9(12):1570–76.

Anderson, Olivia S., Karilyn E. Sant, and Dana C. Dolinoy. 2012. “Nutrition and Epigenetics: An Interplay of Dietary Methyl Donors, One-Carbon Metabolism and DNA Methylation.” *The Journal of Nutritional Biochemistry* 23(8):853–59.

Angelini, Francesco, Francesca Pagano, Antonella Bordin, Marika Milan, Isotta Chimenti, Mariangela Peruzzi, Valentina Valenti, Antonino Marullo, Leonardo Schirone, Silvia Palmerio, Sebastiano Sciarretta, Colin E. Murdoch, Giacomo Frati, and Elena De Falco. 2017. “The Impact of Environmental Factors in Influencing Epigenetics Related to Oxidative States in the Cardiovascular System.” *Oxidative Medicine and Cellular Longevity* 2017(Cvd):2712751.

Annunziato, A. 2008. “DNA Packaging: Nucleosomes and Chromatin.” *Nature Education* 1(1):26.

Aran, Dvir, Gidon Toperoff, Michael Rosenberg, and Asaf Hellman. 2011. “Replication Timing-Related and Gene Body-Specific Methylation of Active Human Genes.” *Human Molecular Genetics* 20(4):670–80.

Arpón, Ana, Fermín I. Milagro, Cristina Razquin, Dolores Corella, Ramón Estruch, Montserrat Fitó, Amelia Marti, Miguel A. Martínez-González, Emilio Ros, Jordi Salas-Salvadó, José-Ignacio Riezu-Boj, and J. Alfredo Martínez. 2017. “Impact of Consuming Extra-Virgin Olive Oil or Nuts within a Mediterranean Diet on DNA Methylation in Peripheral White Blood Cells within the PREDIMED-Navarra

Randomized Controlled Trial: A Role for Dietary Lipids." *Nutrients* 10(1).
Aslibekyan, Stella, Howard W. Wiener, Peter J. Havel, Kimber L. Stanhope, Diane M. O'Brien, Scarlett E. Hopkins, Devin M. Absher, Hemant K. Tiwari, and Bert B. Boyer. 2014. "DNA Methylation Patterns Are Associated with N-3 Fatty Acid Intake in Yup'ik People." *The Journal of Nutrition* 144(4):425–30.
Atamaniuk, Johanna, Claudia Vidotto, Harald Tschan, Norbert Bachl, Karl M. Stuhlmeier, and Mathias M. Müller. 2004. "Increased Concentrations of Cell-Free Plasma DNA after Exhaustive Exercise." *Clinical Chemistry* 50(9):1668–70.
Baccarelli, Andrea, Robert Wright, Valentina Bollati, Augusto Litonjua, Antonella Zanobetti, Letizia Tarantini, David Sparrow, Pantel Vokonas, and Joel Schwartz. 2010. "Ischemic Heart Disease and Stroke in Relation to Blood DNA Methylation." *Epidemiology (Cambridge, Mass.)* 21(6):819–28.
Bajpeyi, Sudip, Jeffrey D. Covington, Erin M. Taylor, Laura K. Stewart, Jose E. Galgani, and Tara M. Henagan. 2017. "Skeletal Muscle PGC1α -1 Nucleosome Position and -260 Nt DNA Methylation Determine Exercise Response and Prevent Ectopic Lipid Accumulation in Men." *Endocrinology* 158(7):2190–99.
Baralle, D. and M. Baralle. 2005. "Splicing in Action: Assessing Disease Causing Sequence Changes." *Journal of Medical Genetics* 42(10):737–48.
Barrès, Romain, Jie Yan, Brendan Egan, Jonas Thue Treebak, Morten Rasmussen, Tomas Fritz, Kenneth Caidahl, Anna Krook, Donal J. O'Gorman, and Juleen R. Zierath. 2012. "Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle." *Cell Metabolism* 15(3):405–11.
Baylin, Stephen B. and Peter A. Jones. 2016. "Epigenetic Determinants of Cancer." *Cold Spring Harbor Perspectives in Biology* 8(9):a019505.
Beavers, Kristen M., Tina E. Brinkley, and Barbara J. Nicklas. 2010. "Effect of Exercise Training on Chronic Inflammation." *Clinica Chimica Acta* 411(11–12):785–93.
Bird, Adrian P. and Alan P. Wolffe. 1999. "Methylation-Induced Repression— Belts, Braces, and Chromatin." *Cell* 99(5):451–54.
Bogdanović, Ozren and Gert Jan C. Veenstra. 2009. "DNA Methylation and Methyl-CpG Binding Proteins: Developmental Requirements and Function." *Chromosoma* 118(5):549–65.
Booth, Frank W., Manu V. Chakravarthy, and Espen E. Spangenburg. 2002. "Exercise and Gene Expression: Physiological Regulation of the Human Genome through Physical Activity." *The Journal of Physiology* 543(2):399–411.
Borg, G. A. 1982. "Psychophysical Bases of Perceived Exertion." *Medicine and Science in Sports and Exercise* 14(5):377–81.
Bouwens, M., O. van de Rest, N. Dellschaft, M. G. Bromhaar, L. C. de Groot, J. M. Geleijnse, M. Muller, and L. a Afman. 2009. "Fish-Oil Supplementation Induces Antiinflammatory Gene Expression Profiles in Human Blood Mononuclear Cells." *American Journal of Clinical Nutrition* 90(2):415–24.
Braconi, Chiara, Nianyuan Huang, and Tushar Patel. 2010. "MicroRNA-Dependent Regulation of DNA Methyltransferase-1 and Tumor Suppressor Gene Expression by Interleukin-6 in Human Malignant Cholangiocytes." *Hepatology* 9(1):NA-NA.
Brancaccio, Paola, Giuseppe Lippi, and Nicola Maffulli. 2010. "Biochemical Markers of Muscular Damage." *Clinical Chemistry and Laboratory Medicine* 48(6):757–67.
Brenet, Fabienne, Michelle Moh, Patricia Funk, Erika Feierstein, Agnes J. Viale, Nicholas D. Socci, and Joseph M. Scandura. 2011. "DNA Methylation of the

First Exon Is Tightly Linked to Transcriptional Silencing." *PLoS ONE* 6(1).
Broadbent, James, Dayle Sampson, Surendran Sabapathy, Luke J. Haseler, Karl-Heinz Wagner, Andrew C. Bulmer, Jonathan M. Peake, and Oliver Neubauer. 2017. "Gene Networks in Skeletal Muscle Following Endurance Exercise Are Coexpressed in Blood Neutrophils and Linked with Blood Inflammation Markers." *Journal of Applied Physiology* 122(4):752–66.
Brookes, Anthony J. 1999. "The Essence of SNPs." *Gene* 234(2):177–86.
Burdge, Graham C., Amanda E. Jones, and Stephen A. Wootton. 2002. "Eicosapentaenoic and Docosapentaenoic Acids Are the Principal Products of α-Linolenic Acid Metabolism in Young Men." *British Journal of Nutrition* 88(04):355–63.
Burdge, Graham C. and Stephen A. Wootton. 2002. "Conversion of α-Linolenic Acid to Eicosapentaenoic, Docosapentaenoic and Docosahexaenoic Acids in Young Women." *British Journal of Nutrition* 88(04):411–20.
Burr, George and Mildred Burr. 1929. "A New Deficiency Disease Produced by the Rigid Exclusion of Fat from the Diet." *Journal of Biological Chemistry* 82(2):345–67.
Burr, George and Mildred Burr. 1930. "On the Nature and Role of the Fatty Acids Essential in Nutrition." *Journal of Biological Chemistry* 86:587–621.
Busquets-Cortés, Carla, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda, and Antoni Pons. 2017. "Training and Acute Exercise Modulates Mitochondrial Dynamics in Football Players' Blood Mononuclear Cells." *European Journal of Applied Physiology* 117(10):1977–87.
Buss, Hendrikje, Timothy P. Chan, Karl B. Sluis, Neil M. Domigan, and Christine C. Winterbourn. 1997. "Protein Carbonyl Measurement by a Sensitive ELISA Method." *Free Radical Biology & Medicine* 23(3):361–66.
Bustin, Stephen and Jim Huggett. 2017. "QPCR Primer Design Revisited." *Biomolecular Detection and Quantification* 14(November):19–28.
Büttner, Petra, Sandy Mosig, Anja Lechtermann, Harald Funke, and Frank C. Mooren. 2007. "Exercise Affects the Gene Expression Profiles of Human White Blood Cells." *Journal of Applied Physiology (Bethesda, Md. : 1985)* 102(1):26–36.
Calder, Philip C. 2004. "N-3 Fatty Acids and Cardiovascular Disease: Evidence Explained and Mechanisms Explored." *Clinical Science (London, England : 1979)* 107:1–11.
Calder, Philip C. 2011. "Fatty Acids and Inflammation: The Cutting Edge between Food and Pharma." *European Journal of Pharmacology* 668(SUPPL. 1):S50–58.
Calder, Philip C. 2012. "Mechanisms of Action of (n-3) Fatty Acids." *The Journal of Nutrition* 142(3):592S-599S.
Calder, Philip C. 2015. "Marine Omega-3 Fatty Acids and Inflammatory Processes: Effects, Mechanisms and Clinical Relevance." *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1851(4):469–84.
Campanelli, Rita, Gabriela Fois, P. Catarsi, Valentina Poletto, Laura Villani, Benedetta Gaia Erba, Luigi Maddaluno, Basilio Jemos, Silvia Salmoiraghi, Paola Guglielmelli, Vittorio Abbonante, Christian Andrea Di Buduo, Alessandra Balduini, Alessandra Iurlo, Giovanni Barosi, Vittorio Rosti, Margherita Massa, A. M. Vannucchi, M. Balliu, N. Bartalucci, C. Bogani, A. Bosi, L. Calabresi, G. Corbizzi Fattori, T. Fanelli, R. Fjerza, F. Gesullo, P. Guglielmelli, C. Mannarelli, L. Merli, A. Pacilli, A. Pancrazzi, C. Paoli, L. Pieri, G. Rotunno, E. Sant'Antonio, G. Barosi, V. Abbonante, A. Balduini, E. Bonetti, R. Campanelli, C. A. Di Buduo,

G. Fois, M. Massa, V. Poletto, V. Rosti, L. Villani, M. Cazzola, I. Ambaglio, P. Bernasconi, C. I. Casetti, S. Catricalà, C. Elena, E. Fugazza, A. Gallì, L. Malcovati, C. Milanesi, C. Pascutto, D. Pietra, F. Ripamonti, M. Rossi, E. Rumi, E. Dejana, F. Breviario, M. Corada, M. Malinverno, A. Rambaldi, G. Chioda, M. L. Ferrari, G. Finazzi, M. C. Finazzi, C. Belotti, C. Boroni, S. Salmoiraghi, A. Amaru, J. Golay, S. Bortoluzzi, A. Bisognin, A. Coppe, C. Saccoman, R. Manfredini, L. Artuso, I. Bernardis, E. Bianchi, M. Montanari, V. Pennucci, Z. Prudente, S. Rontauroli, C. Rossi, S. Ruberti, S. Salati, E. Tagliafico, E. Tenedini, and R. Zini. 2016. “Tie2 Expressing Monocytes in the Spleen of Patients with Primary Myelofibrosis.” *PLoS ONE* 11(6):1–15.

Capomaccio, Stefano, Katia Cappelli, Giacomo Spinsanti, Marzia Mencarelli, Michela Muscettola, Michela Felicetti, Andrea Verini Supplizi, and Marco Bonifazi. 2011. “Athletic Humans and Horses: Comparative Analysis of Interleukin-6 (IL-6) and IL-6 Receptor (IL-6R) Expression in Peripheral Blood Mononuclear Cells in Trained and Untrained Subjects at Rest.” *BMC Physiology* 11(1):3.

Carty, J. L., R. Bevan, H. Waller, N. Mistry, M. Cooke, J. Lunec, and H. R. Griffiths. 2000. “The Effects of Vitamin C Supplementation on Protein Oxidation in Healthy Volunteers.” *Biochemical and Biophysical Research Communications* 273(2):729–35.

Casillas, Mark A., Nadejda Lopatina, Lucy G. Andrews, and Trygve O. Tollefsbol. 2003. “Transcriptional Control of the DNA Methyltransferases Is Altered in Aging and Neoplastically-Transformed Human Fibroblasts.” *Molecular and Cellular Biochemistry* 252(1–2):33–43.

Ceci, Roberta, Maria Reyes Beltran Valls, Guglielmo Duranti, Ivan Dimauro, Federico Quaranta, Monica Pittaluga, Stefania Sabatini, Paolo Caserotti, Paolo Parisi, Attilio Parisi, and Daniela Caporossi. 2014. “Oxidative Stress Responses to a Graded Maximal Exercise Test in Older Adults Following Explosive-Type Resistance Training.” *Redox Biology* 2:65–72.

Cesari, M., B. W. J. H. Penninx, M. Pahor, F. Lauretani, A. M. Corsi, G. R. Williams, J. M. Guralnik, and L. Ferrucci. 2004. “Inflammatory Markers and Physical Performance in Older Persons: The InCHIANTI Study.” *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 59(3):M242–48.

Chakraborty, Nabarun, Seid Muhie, Raina Kumar, Aarti Gautam, Seshamalini Srinivasan, Bintu Sowe, George Dimitrov, Stacy-Ann Miller, Marti Jett, and Rasha Hammamieh. 2017. “Contributions of Polyunsaturated Fatty Acids (PUFA) on Cerebral Neurobiology: An Integrated Omics Approach with Epigenomic Focus.” *The Journal of Nutritional Biochemistry* 42:84–94.

Chen, Chun-Chang, Keh-Yang Wang, and Che-Kun Shen. 2013. “DNA 5-Methylcytosine Demethylation Activities of the Mammalian DNA Methyltransferases.” *The Journal of Biological Chemistry* 288(13):9084–91.

Chen, Chun-Chang, Keh-Yang Wang, and Che-Kun James Shen. 2012. “The Mammalian de Novo DNA Methyltransferases DNMT3A and DNMT3B Are Also DNA 5-Hydroxymethylcytosine Dehydroxymethylases.” *The Journal of Biological Chemistry* 287(40):33116–21.

Chen, Richard Z., Ulf Pettersson, Caroline Beard, Laurie Jackson-Grusby, and Rudolf Jaenisch. 1998. “DNA Hypomethylation Leads to Elevated Mutation Rates.” *Nature* 395(6697):89–93.

Chen, Shuen-Ei, Bingwen Jin, and Yi-Ping Li. 2007. “TNF-Alpha Regulates

Myogenesis and Muscle Regeneration by Activating P38 MAPK." *American Journal of Physiology. Cell Physiology* 292(5):C1660-71.
Chen, Taiping, Yoshihide Ueda, Jonathan E. Dodge, Zhenjuan Wang, and En Li. 2003. "Establishment and Maintenance of Genomic Methylation Patterns in Mouse Embryonic Stem Cells by Dnmt3a and Establishment and Maintenance of Genomic Methylation Patterns in Mouse Embryonic Stem Cells by Dnmt3a and Dnmt3b." *Society* 23(16):5594–5605.
Chilton, Floyd, Robert Murphy, Bryan Wilson, Susan Sergeant, Hannah Ainsworth, Michael Seeds, and Rasika Mathias. 2014. "Diet-Gene Interactions and PUFA Metabolism: A Potential Contributor to Health Disparities and Human Diseases." *Nutrients* 6(5):1993–2022.
Cho, Hyekyung P., Manabu Nakamura, and Steven D. Clarke. 1999. "Cloning, Expression, and Fatty Acid Regulation of the Human Δ-5 Desaturase." *Journal of Biological Chemistry* 274(52):37335–39.
Cho, Hyekyung P, Manabu T. Nakamura, and Steven D. Clarke. 1999. "Cloning , Expression , and Nutritional Regulation of the Mammalian ⌬ -6 Desaturase *." 274(1):471–77.
Chodavarapu, Ramakrishna K., Suhua Feng, Yana V Bernatavichute, Pao-Yang Chen, Hume Stroud, Yanchun Yu, Jonathan a Hetzel, Frank Kuo, Jin Kim, Shawn J. Cokus, David Casero, Maria Bernal, Peter Huijser, Amander T. Clark, Ute Krämer, Sabeeha S. Merchant, Xiaoyu Zhang, Steven E. Jacobsen, and Matteo Pellegrini. 2010. "Relationship between Nucleosome Positioning and DNA Methylation." *Nature* 466(7304):388–92.
Choi, S. W. and S. Friso. 2010. "Epigenetics: A New Bridge between Nutrition and Health." *Advances in Nutrition: An International Review Journal* 1(1):8–16.
Christiansen, Tore, Søren K. Paulsen, Jens M. Bruun, Steen B. Pedersen, and Bjørn Richelsen. 2010. "Exercise Training versus Diet-Induced Weight-Loss on Metabolic Risk Factors and Inflammatory Markers in Obese Subjects: A 12-Week Randomized Intervention Study." *American Journal of Physiology. Endocrinology and Metabolism* 298(4):E824-31.
Clancey, S. and W. Brown. 2008. "Translation: DNA to MRNA to Protein." *Nature Education* 1(1):101.
Clarke-Harris, Rebecca, Terence J. Wilkin, Joanne Hosking, Jonathan Pinkney, Alison N. Jeffery, Brad S. Metcalf, Keith M. Godfrey, Linda D. Voss, Karen A. Lillycrop, and Graham C. Burdge. 2014. "PGC1α Promoter Methylation in Blood at 5-7 Years Predicts Adiposity from 9 to 14 Years (EarlyBird 50)." *Diabetes* 63(7):2528–37.
Clarkson, Priscilla M. and Monica J. Hubal. 2002. "Exercise-Induced Muscle Damage in Humans." *American Journal of Physical Medicine & Rehabilitation* 81(11 Suppl):S52-69.
Coffey, Vernon G. and John A. Hawley. 2017. "Concurrent Exercise Training: Do Opposites Distract?" *The Journal of Physiology* 595(9):2883–96.
Coffey, Vernon G. and John a Hawley. 2007. "The Molecular Basis of Training Adaptation." *Sports Medicine* 37(9):737–63.
Compere, S. J. and R. D. Palmiter. 1981. "DNA Methylation Controls the Inducibility of the Mouse Metallothionein-I Gene in Lymphoid Cells." *Cell* 25(1):233–40.
Connolly, Peter H., Vincent J. Caiozzo, Frank Zaldivar, Dan Nemet, Jennifer Larson, She-Pin Hung, J. Denis Heck, G. Wesley Hatfield, and Dan M. Cooper. 2004. "Effects of Exercise on Gene Expression in Human Peripheral Blood Mononuclear Cells." *Journal of Applied Physiology (Bethesda, Md. : 1985)*

97(4):1461–69.
Corominas-Faja, Bruna, Elisabet Cuyàs, Jesús Lozano-Sánchez, Sílvia Cufí, Sara Verdura, Salvador Fernández-Arroyo, Isabel Borrás-Linares, Begoña Martin-Castillo, Ángel G. Martin, Ruth Lupu, Alfons Nonell-Canals, Melchor Sanchez-Martinez, Vicente Micol, Jorge Joven, Antonio Segura-Carretero, and Javier A. Menendez. 2018. “Extra-Virgin Olive Oil Contains a Metabolo-Epigenetic Inhibitor of Cancer Stem Cells.” *Carcinogenesis* 39(4):601–13.
Cui, Tao, Austin G. Hester, Michael C. Seeds, Elaheh Rahbar, Timothy D. Howard, Susan Sergeant, and Floyd H. Chilton. 2016. “Impact of Genetic and Epigenetic Variations Within the FADS Cluster on the Composition and Metabolism of Polyunsaturated Fatty Acids in Prostate Cancer.” *The Prostate* 76(13):1182–91.
D’Amore, Simona, Michele Vacca, Marica Cariello, Giusi Graziano, Andria D’Orazio, Roberto Salvia, Rosa Cinzia Sasso, Carlo Sabbà, Giuseppe Palasciano, and Antonio Moschetta. 2016. “Genes and MiRNA Expression Signatures in Peripheral Blood Mononuclear Cells in Healthy Subjects and Patients with Metabolic Syndrome after Acute Intake of Extra Virgin Olive Oil.” *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1861(11):1671–80.
Damas, Felipe, Stuart Phillips, Felipe Cassaro Vechin, and Carlos Ugrinowitsch. 2015. “A Review of Resistance Training-Induced Changes in Skeletal Muscle Protein Synthesis and Their Contribution to Hypertrophy.” *Sports Medicine* 45(6):801–7.
Dedeurwaerder, Sarah, Matthieu Defrance, Martin Bizet, Emilie Calonne, Gianluca Bontempi, and François Fuks. 2014. “A Comprehensive Overview of Infinium HumanMethylation450 Data Processing.” *Briefings in Bioinformatics* 15(6):929–41.
Delaney, Colin, Sanjay K. Garg, and Raymond Yung. 2015. “Analysis of DNA Methylation by Pyrosequencing.” *Methods in Molecular Biology (Clifton, N.J.)* 1343:249–64.
Denham, Joshua, Francine Z. Marques, Emma L. Bruns, Brendan J. O’Brien, and Fadi J. Charchar. 2016. “Epigenetic Changes in Leukocytes after 8 Weeks of Resistance Exercise Training.” *European Journal of Applied Physiology* 116(6):1245–53.
Denham, Joshua, B. O’Brien, Jack T. Harvey, and Fadi J. Charchar. 2015. “Genome-Wide Sperm DNA Methylation Changes after 3 Months of Exercise Training in Humans.” *Epigenomics* 7(5):717–31.
Denham, Joshua, B. O’Brien, Francine Z. Marques, and Fadi J. Charchar. 2015. “Changes in the Leukocyte Methylome and Its Effect on Cardiovascular-Related Genes after Exercise.” *Journal of Applied Physiology* 118(4):475–88.
van Dijk, Susan J., Jing Zhou, Timothy J. Peters, Michael Buckley, Brodie Sutcliffe, Yalchin Oytam, Robert A. Gibson, Andrew McPhee, Lisa N. Yelland, Maria Makrides, Peter L. Molloy, and Beverly S. Muhlhausler. 2016. “Effect of Prenatal DHA Supplementation on the Infant Epigenome: Results from a Randomized Controlled Trial.” *Clinical Epigenetics* 8(1):1–13.
Dill, D. B. and D. L. Costill. 1974. “Calculation of Percentage Changes in Volumes of Blood, Plasma, and Red Cells in Dehydration.” *Journal of Applied Physiology* 37(2):247–48.
Dimauro, Ivan, Mattia Scalabrin, Cristina Fantini, Elisa Grazioli, Maria Reyes Beltran Valls, Neri Mercatelli, Attilio Parisi, Stefania Sabatini, Luigi Di Luigi, and Daniela Caporossi. 2016. “Resistance Training and Redox Homeostasis: Correlation with Age-Associated Genomic Changes.” *Redox Biology* 10:34–44.

Dong, Xianjun and Zhiping Weng. 2013. “The Correlation between Histone Modifications and Gene Expression.” *Epigenomics* 5(2):113–16.
Donkin, Ida and Romain Barrès. 2018. “Sperm Epigenetics and Influence of Environmental Factors.” *Molecular Metabolism* 14(February):1–11.
Duggan, Catherine, Liren Xiao, Mary Beth Terry, and Anne McTiernan. 2014. “No Effect of Weight Loss on LINE-1 Methylation Levels in Peripheral Blood Leukocytes from Postmenopausal Overweight Women.” *Obesity (Silver Spring, Md.)* 22(9):2091–96.
Dumas-Mallet, Estelle, Katherine S. Button, Thomas Boraud, Francois Gonon, and Marcus R. Munafò. 2017. “Low Statistical Power in Biomedical Science: A Review of Three Human Research Domains.” *Royal Society Open Science* 4(2):160254.
Duursma, Anja M., Martijn Kedde, Mariette Schrier, Carlos le Sage, and Reuven Agami. 2008. “MiR-148 Targets Human DNMT3b Protein Coding Region.” *RNA (New York, N.Y.)* 14(5):872–77.
Dyrvig, Mads, Per Qvist, Jacek Lichota, Knud Larsen, Mette Nyegaard, Anders D. Børglum, and Jane H. Christensen. 2017. “DNA Methylation Analysis of BRD1 Promoter Regions and the Schizophrenia Rs138880 Risk Allele.” *PLoS ONE* 12(1):1–19.
Eads, C. A., K. D. Danenberg, K. Kawakami, L. B. Saltz, C. Blake, D. Shibata, P. V Danenberg, and P. W. Laird. 2000. “MethyLight: A High-Throughput Assay to Measure DNA Methylation.” *Nucleic Acids Research* 28(8):E32.
Egan, Brendan, Brian P. Carson, Pablo M. Garcia-Roves, Alexander V Chibalin, Fiona M. Sarsfield, Niall Barron, Noel McCaffrey, Niall M. Moyna, Juleen R. Zierath, and Donal J. O’Gorman. 2010. “Exercise Intensity-Dependent Regulation of Peroxisome Proliferator-Activated Receptor Coactivator-1 MRNA Abundance Is Associated with Differential Activation of Upstream Signalling Kinases in Human Skeletal Muscle.” *The Journal of Physiology* 588(Pt 10):1779–90.
Egan, Brendan and Juleen R. Zierath. 2013. “Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation.” *Cell Metabolism* 17(2):162–84.
Egger, Gerda, Gangning Liang, Ana Aparicio, and Peter A. Jones. 2004. “Epigenetics in Human Disease and Prospects for Epigenetic Therapy.” *Nature* 429(6990):457–63.
Ehrlich, Melanie. 2009. “DNA Hypomethylation in Cancer Cells.” *Epigenomics* 1(2):239–59.
Ehrlich, Melanie, Miguel A. Gama-Sosa, Lan Hsiang Huang, Rose Marie Midgett, Kenneth C. Kuo, Roy A. Mccune, and Charles Gehrke. 1982. “Amount and Distribution of 5-Methylcytosine in Human DNA from Different Types of Tissues or Cells.” *Nucleic Acids Research* 10(8):2709–21.
Eisele, Petra S., Regula Furrer, Markus Beer, and Christoph Handschin. 2015. “The PGC-1 Coactivators Promote an Anti-Inflammatory Environment in Skeletal Muscle in Vivo.” *Biochemical and Biophysical Research Communications* 464(3):692–97.
Eisele, Petra S., Silvia Salatino, Jens Sobek, Michael O. Hottiger, and Christoph Handschin. 2013. “The Peroxisome Proliferator-Activated Receptor γ Coactivator 1α/β (PGC-1) Coactivators Repress the Transcriptional Activity of NF-KB in Skeletal Muscle Cells.” *Journal of Biological Chemistry* 288(4):2246–60.

Encode Consortium. 2012. “An Integrated Encyclopedia of DNA Elements in the Human Genome.” *Nature* 489(7414):57–74.
Estruch, Ramón, Emilio Ros, Jordi Salas-Salvadó, Maria-Isabel Covas, Dolores Corella, Fernando Arós, Enrique Gómez-Gracia, Valentina Ruiz-Gutiérrez, Miquel Fiol, José Lapetra, Rosa M. Lamuela-Raventos, Lluís Serra-Majem, Xavier Pintó, Josep Basora, Miguel A. Muñoz, José V Sorlí, J. Alfredo Martínez, Montserrat Fitó, Alfredo Gea, Miguel A. Hernán, Miguel A. Martínez-González, and PREDIMED Study Investigators. 2018. “Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts.” *The New England Journal of Medicine* 378(25):e34.
Fabbri, Muller, Ramiro Garzon, Amelia Cimmino, Zhongfa Liu, Nicola Zanesi, Elisa Callegari, Shujun Liu, Hansjuerg Alder, Stefan Costinean, Cecilia Fernandez-Cymering, Stefano Volinia, Gulnur Guler, Carl D. Morrison, Kenneth K. Chan, Guido Marcucci, George A. Calin, Kay Huebner, and Carlo M. Croce. 2007. “MicroRNA-29 Family Reverts Aberrant Methylation in Lung Cancer by Targeting DNA Methyltransferases 3A and 3B.” *Proceedings of the National Academy of Sciences of the United States of America* 104(40):15805–10.
Fazzari, Melissa J. and John M. Greally. 2004. “Epigenomics: Beyond CpG Islands.” *Nature Reviews Genetics* 5(6):446–55.
Ferrer, Miguel D., Pedro Tauler, Antoni Sureda, Josep A. Tur, and Antoni Pons. 2009. “Antioxidant Regulatory Mechanisms in Neutrophils and Lymphocytes after Intense Exercise.” *Journal of Sports Sciences* 27(1):49–58.
Flynn, Michael G., Brian K. McFarlin, and Melissa M. Markofski. 2007. “State of the Art Reviews: The Anti-Inflammatory Actions of Exercise Training.” *American Journal of Lifestyle Medicine* 1(3):220–35.
Fontes, João D., Faisal Rahman, Sean Lacey, Martin G. Larson, Ramachandran S. Vasan, Emelia J. Benjamin, William S. Harris, and Sander J. Robins. 2015. “Red Blood Cell Fatty Acids and Biomarkers of Inflammation: A Cross-Sectional Study in a Community-Based Cohort.” *Atherosclerosis* 240:431–36.
Foran, E., M. M. Garrity-Park, C. Mureau, J. Newell, T. C. Smyrk, P. J. Limburg, and L. J. Egan. 2010. “Upregulation of DNA Methyltransferase-Mediated Gene Silencing, Anchorage-Independent Growth, and Migration of Colon Cancer Cells by Interleukin-6.” *Molecular Cancer Research* 8(4):471–81.
Fraga, M. F., E. Ballestar, M. F. Paz, S. Ropero, F. Setien, M. L. Ballestar, D. Heine-Suner, J. C. Cigudosa, M. Urioste, J. Benitez, M. Boix-Chornet, A. Sanchez-Aguilera, C. Ling, E. Carlsson, P. Poulsen, A. Vaag, Z. Stephan, T. D. Spector, Y. Z. Wu, C. Plass, and M. Esteller. 2005. “From The Cover: Epigenetic Differences Arise during the Lifetime of Monozygotic Twins.” *Proceedings of the National Academy of Sciences* 102(30):10604–9.
Di Francesco, Andrea, Beatrice Arosio, Anastasia Falconi, Maria Vittoria Micioni Di Bonaventura, Mohsen Karimi, Daniela Mari, Martina Casati, Mauro Maccarrone, and Claudio D’Addario. 2015. “Global Changes in DNA Methylation in Alzheimer’s Disease Peripheral Blood Mononuclear Cells.” *Brain, Behavior, and Immunity* 45:139–44.
Frías-Lasserre, Daniel and Cristian A. Villagra. 2017. “The Importance of NcRNAs as Epigenetic Mechanisms in Phenotypic Variation and Organic Evolution.” *Frontiers in Microbiology* 8(DEC):1–13.
Friso, Simonetta, Silvia Udali, Patrizia Guarini, Camilla Pellegrini, Patrizia Pattini, Sara Moruzzi, Domenico Girelli, Francesca Pizzolo, Nicola Martinelli, Roberto Corrocher, Oliviero Olivieri, and Sang-Woon Choi. 2013. “Global DNA

Hypomethylation in Peripheral Blood Mononuclear Cells as a Biomarker of Cancer Risk." *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology* 22(3):348–55.

Gao, Xu Dong, Jian Hui Qu, Xiu Juan Chang, Yin Ying Lu, Wen Lin Bai, Hong Wang, Zhong Xian Xu, Lin Jing An, Chun Ping Wang, Zhen Zeng, and Yong Ping Yang. 2014. "Hypomethylation of Long Interspersed Nuclear Element-1 Promoter Is Associated with Poor Outcomes for Curative Resected Hepatocellular Carcinoma." *Liver International* 34(1):136–46.

Garber, Carol Ewing, Bryan Blissmer, Michael R. Deschenes, Barry A. Franklin, Michael J. Lamonte, I. Min Lee, David C. Nieman, David P. Swain, and American College of Sports Medicine. 2011. "American College of Sports Medicine Position Stand. Quantity and Quality of Exercise for Developing and Maintaining Cardiorespiratory, Musculoskeletal, and Neuromotor Fitness in Apparently Healthy Adults: Guidance for Prescribing Exercise." *Medicine and Science in Sports and Exercise* 43(7):1334–59.

Gardiner-Garden, M. and M. Frommer. 1987. "CpG Islands in Vertebrate Genomes." *Journal of Molecular Biology* 196(2):261–82.

Garzon, Ramiro, Shujun Liu, Muller Fabbri, Zhongfa Liu, Catherine E. A. Heaphy, Elisa Callegari, Sebastian Schwind, Jiuxia Pang, Jianhua Yu, Natarajan Muthusamy, Violaine Havelange, Stefano Volinia, William Blum, Laura J. Rush, Danilo Perrotti, Michael Andreeff, Clara D. Bloomfield, John C. Byrd, Kenneth Chan, Lai-Chu Wu, Carlo M. Croce, and Guido Marcucci. 2009. "MicroRNA-29b Induces Global DNA Hypomethylation and Tumor Suppressor Gene Reexpression in Acute Myeloid Leukemia by Targeting Directly DNMT3A and 3B and Indirectly DNMT1." *Blood* 113(25):6411–18.

Genereux, Diane P., Winslow C. Johnson, Alice F. Burden, Reinhard Stöger, and Charles D. Laird. 2009. "Errors in the Bisulfite Conversion of DNA: Modulating Inappropriate- and Failed-Conversion Frequencies." *Nucleic Acids Research* 37(15):5235.

Gillberg, Linn, Stine C. Jacobsen, Tina Rönn, Charlotte Brøns, and Allan Vaag. 2014. "PPARGC1A DNA Methylation in Subcutaneous Adipose Tissue in Low Birth Weight Subjects - Impact of 5 Days of High-Fat Overfeeding." *Metabolism: Clinical and Experimental* 63(2):263–71.

Gjevestad, Gyrd O., Håvard Hamarsland, Truls Raastad, Inger Ottestad, Jacob J. Christensen, Kristin Eckardt, Christian A. Drevon, Anne S. Biong, Stine M. Ulven, and Kirsten B. Holven. 2017. "Gene Expression Is Differentially Regulated in Skeletal Muscle and Circulating Immune Cells in Response to an Acute Bout of High-Load Strength Exercise." *Genes and Nutrition* 12(1):1–11.

Gjevestad, Gyrd O., Kirsten B. Holven, and Stine M. Ulven. 2015. "Effects of Exercise on Gene Expression of Inflammatory Markers in Human Peripheral Blood Cells: A Systematic Review." *Current Cardiovascular Risk Reports* 9(7):34.

Glaser, Claudia, Joachim Heinrich, and Berthold Koletzko. 2010. "Role of FADS1 and FADS2 Polymorphisms in Polyunsaturated Fatty Acid Metabolism." *Metabolism: Clinical and Experimental* 59(7):993–99.

Gleeson, Michael, Nicolette C. Bishop, David J. Stensel, Martin R. Lindley, Sarabjit S. Mastana, and Myra A. Nimmo. 2011. "The Anti-Inflammatory Effects of Exercise: Mechanisms and Implications for the Prevention and Treatment of Disease." *Nature Reviews. Immunology* 11(9):607–15.

Gluckman, Peter D. and Mark A. Hanson. 2004. “Developmental Origins of Disease Paradigm: A Mechanistic and Evolutionary Perspective.” *Pediatric Research* 56(3):311–17.
Grazioli, Elisa, Ivan Dimauro, Neri Mercatelli, Guan Wang, Yannis Pitsiladis, Luigi Di Luigi, and Daniela Caporossi. 2017. “Physical Activity in the Prevention of Human Diseases: Role of Epigenetic Modifications.” *BMC Genomics* 18(Suppl 8):802.
Hackett, Jamie A. and M. Azim Surani. 2013. “DNA Methylation Dynamics during the Mammalian Life Cycle.” *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 368(1609):20110328.
Hammond, Scott M. 2015. “An Overview of MicroRNAs.” *Advanced Drug Delivery Reviews* 87(3):3–14.
Hancock, Chad R., Dong-Ho Han, May Chen, Shin Terada, Toshihiro Yasuda, David C. Wright, and John O. Holloszy. 2008. “High-Fat Diets Cause Insulin Resistance despite an Increase in Muscle Mitochondria.” *Proceedings of the National Academy of Sciences of the United States of America* 105(22):7815–20.
Handschin, Christoph and Bruce M. Spiegelman. 2008. “The Role of Exercise and PGC1alpha in Inflammation and Chronic Disease.” *Nature* 454(7203):463–69.
Harrington, Colleen T., Elaine I. Lin, Matthew T. Olson, and James R. Eshleman. 2013. “Fundamentals of Pyrosequencing.” *Archives of Pathology and Laboratory Medicine* 137(9):1296–1303.
Hawley, John A. 2002. “Proceedings of the Australian Physiological and Pharmacological Society Symposium : Integrative Physiology of Exercise ADAPTATIONS OF SKELETAL MUSCLE TO PROLONGED , INTENSE ENDURANCE TRAINING.” *Clinical and Experimental Pharmacology and Physiology* 29(November 2000):218–22.
He, Feng, Juan Li, Zewen Liu, Chia-Chen Chuang, Wenge Yang, and Li Zuo. 2016. “Redox Mechanism of Reactive Oxygen Species in Exercise.” *Frontiers in Physiology* 7(NOV):486.
Herman, James G., Jeremy R. Graff, Sanna Myohanen, Barry D. Nelkin, and Stephen B. Baylin. 1996. “Methylation-Specific PCR: A Novel PCR Assay for Methylation Status of CpG Islands (DNA Methylation/Tumor Suppressor Genes/Pl6/P15).” *Proceedings of the National Academy of Sciences of the United States of America* 93(September):9821–26.
Hermsdorff, H. H., M. L. Mansego, J. Campión, F. I. Milagro, M. A. Zulet, and J. A. Martínez. 2013. “TNF-Alpha Promoter Methylation in Peripheral White Blood Cells: Relationship with Circulating TNFα, Truncal Fat and n-6 PUFA Intake in Young Women.” *Cytokine* 64(1):265–71.
Hernández, Hernán G., M. Yat Tse, Stephen C. Pang, Humberto Arboleda, and Diego a. Forero. 2013. “Optimizing Methodologies for PCR-Based DNA Methylation Analysis.” *BioTechniques* 55(4):181–97.
Hodge, David R., Weihua Xiao, Peter A. Clausen, Gisela Heidecker, Moshe Szyf, and William L. Farrar. 2001. “Interleukin-6 Regulation of the Human DNA Methyltransferase (HDNMT) Gene in Human Erythroleukemia Cells.” *The Journal of Biological Chemistry* 276(43):39508–11.
Hoile, Samuel P., Rebecca Clarke-Harris, Rae-Chi Huang, Philip C. Calder, Trevor a. Mori, Lawrence J. Beilin, Karen a. Lillycrop, and Graham C. Burdge. 2014. “Supplementation with N-3 Long-Chain Polyunsaturated Fatty Acids or Olive Oil in Men and Women with Renal Disease Induces Differential Changes in the

DNA Methylation of FADS2 and ELOVL5 in Peripheral Blood Mononuclear Cells." edited by W.-H. Schunck. *PloS One* 9(10):e109896.
Hoile, Samuel P, Rebecca Clarke-Harris, Rae-Chi Huang, Philip C. Calder, Trevor A. Mori, Lawrence J. Beilin, Karen A. Lillycrop, and Graham C. Burdge. 2014. "Supplementation with N-3 Long-Chain Polyunsaturated Fatty Acids or Olive Oil in Men and Women with Renal Disease Induces Differential Changes in the DNA Methylation of FADS2 and ELOVL5 in Peripheral Blood Mononuclear Cells." *PloS One* 9(10):e109896.
Horsburgh, Steven, Stephen Todryk, Christopher Toms, Colin N. Moran, and Les Ansley. 2015. "Exercise-Conditioned Plasma Attenuates Nuclear Concentrations of DNA Methyltransferase 3B in Human Peripheral Blood Mononuclear Cells." *Physiological Reports* 3(12):1–10.
Howley, E. T., D. R. Bassett, and H. G. Welch. 1995. "Criteria for Maximal Oxygen Uptake: Review and Commentary." *Medicine and Science in Sports and Exercise* 27(9):1292–1301.
Illingworth, Robert S. and Adrian P. Bird. 2009. "CpG Islands - 'A Rough Guide.'" *FEBS Letters* 583(11):1713–20.
Itariu, Bianca K., Maximilian Zeyda, Eva E. Hochbrugger, Angelika Neuhofer, Gerhard Prager, Karin Schindler, Arthur Bohdjalian, Daniel Mascher, Suman Vangala, Michael Schranz, Michael Krebs, Martin G. Bischof, and Thomas M. Stulnig. 2012. "Long-Chain n-3 PUFAs Reduce Adipose Tissue and Systemic Inflammation in Severely Obese Nondiabetic Patients: A Randomized Controlled Trial." *American Journal of Clinical Nutrition* 96(5):1137–49.
Izzi, Benedetta, Alexandra M. Binder, and Karin B. Michels. 2014. "Pyrosequencing Evaluation of Widely Available Bisulfite Conversion Methods: Considerations for Application." *Medical Epigenetics* 2(1):28–36.
Jaffe, Andrew E. and Rafael A. Irizarry. 2014. "Accounting for Cellular Heterogeneity Is Critical in Epigenome-Wide Association Studies." *Genome Biology* 15(2):R31.
Jaiswal, Deepika, Sameer Trivedi, Neeraj K. Agrawal, and Kiran Singh. 2015. "Association of the Patterns of Global DNA Methylation and Expression Analysis of DNA Methyltransferases in Impaired Spermatogenic Patients." *Asian Pacific Journal of Reproduction* 4(4):262–65.
Jeukendrup, A., W. H. Saris, F. Brouns, and A. D. Kester. 1996. "A New Validated Endurance Performance Test." *Medicine and Science in Sports and Exercise* 28(2):266–70.
Jiang, Wanlin, Devendra K. Agrawal, and Chandra S. Boosani. 2018. "Cell-Specific Histone Modifications in Atherosclerosis (Review)." *Molecular Medicine Reports* 18(2):1215–24.
Jin, Zelin and Yun Liu. 2018. "DNA Methylation in Human Diseases." *Genes and Diseases* 5(1):1–8.
Jones, Meaghan J., Sumaiya A. Islam, Rachel D. Edgar, and Michael S. Kobor. 2017. "Adjusting for Cell Type Composition in DNA Methylation Data Using a Regression-Based Approach." *Methods in Molecular Biology (Clifton, N.J.)* 1589(1341):99–106.
Jones, Peter A. 2012. "Functions of DNA Methylation: Islands, Start Sites, Gene Bodies and Beyond." *Nature Reviews Genetics* 13(7):484–92.
Joyce, Brian T., Tao Gao, Yinan Zheng, Lei Liu, Wei Zhang, Qi Dai, Martha J. Shrubsole, Elizabeth A. Hibler, Massimo Cristofanilli, Hu Zhang, Hushan Yang, Pantel Vokonas, Laura Cantone, Joel Schwartz, Andrea Baccarelli, and Lifang Hou. 2016. "Prospective Changes in Global DNA Methylation and Cancer

Incidence and Mortality.” *British Journal of Cancer* 115(4):465–72.
Kaminsky, Leonard A., Ross Arena, and Jonathan Myers. 2015. “Reference Standards for Cardiorespiratory Fitness Measured with Cardiopulmonary Exercise Testing Data from the Fitness Registry and the Importance of Exercise National Database.” *Mayo Clinic Proceedings* 90(11):1515–23.
Kanherkar, Riya R., Naina Bhatia-Dey, and Antonei B. Csoka. 2014. “Epigenetics across the Human Lifespan.” *Frontiers in Cell and Developmental Biology* 2(September):1–19.
Kareta, Michael S., Zaida M. Botello, Joshua J. Ennis, Christina Chou, and Frédéric Chédin. 2006. “Reconstitution and Mechanism of the Stimulation of de Novo Methylation by Human DNMT3L.” *Journal of Biological Chemistry* 281(36):25893–902.
Karimi, Mohsen, Sofia Johansson, Dirk Stach, Martin Corcoran, Dan Grandér, Martin Schalling, Georgy Bakalkin, Frank Lyko, Catharina Larsson, and Tomas J. Ekström. 2006. “LUMA (LUminometric Methylation Assay)--a High Throughput Method to the Analysis of Genomic DNA Methylation.” *Experimental Cell Research* 312(11):1989–95.
Karimi, Mohsen, Inger Vedin, Yvonne Freund Levi, Hans Basun, Gerd Faxén Irving, Maria Eriksdotter, Lars-Olof Olof Wahlund, Marianne Schultzberg, Erik Hjorth, Tommy Cederholm, Jan Palmblad, Yvonne Freund Levi, Hans Basun, Gerd Faxén Irving, Maria Eriksdotter, Lars-Olof Olof Wahlund, Marianne Schultzberg, Erik Hjorth, Tommy Cederholm, and Jan Palmblad. 2017. “DHA-Rich n–3 Fatty Acid Supplementation Decreases DNA Methylation in Blood Leukocytes: The OmegAD Study.” *American Journal of Clinical Nutrition* 106(4):1157–65.
Kaur, Narinder, Vishal Chugh, and Anil K. Gupta. 2014. “Essential Fatty Acids as Functional Components of Foods- a Review.” *Journal of Food Science and Technology* 51(10):2289–2303.
Kaut, Oliver, Alfredo Ramirez, Heike Pieper, Ina Schmitt, Frank Jessen, and Ullrich Wüllner. 2014. “DNA Methylation of the TNF-α Promoter Region in Peripheral Blood Monocytes and the Cortex of Human Alzheimer’s Disease Patients.” *Dementia and Geriatric Cognitive Disorders* 38(1–2):10–15.
Kawabata, Fuminori, Mitsuo Neya, Kei Hamazaki, Yuya Watanabe, Satoru Kobayashi, and Tomoko Tsuji. 2014. “Supplementation with Eicosapentaenoic Acid-Rich Fish Oil Improves Exercise Economy and Reduces Perceived Exertion during Submaximal Steady-State Exercise in Normal Healthy Untrained Men.” *Bioscience, Biotechnology, and Biochemistry* 78(12):2081–88.
Kim, Kwi Baek. 2014. “Effect of Different Training Mode on Interleukin-6 (IL-6) and C-Reactive Protein (CRP) in Type 2 Diabetes Mellitus (T2DM) Patients.” *Journal of Exercise Nutrition & Biochemistry* 18(4):371–78.
Kim, Myungjin, Tiffany I. Long, Kazuko Arakawa, Renwei Wang, Mimi C. Yu, and Peter W. Laird. 2010. “DNA Methylation as a Biomarker for Cardiovascular Disease Risk.” *PLoS ONE* 5(3):1–8.
King-Himmelreich, Tanya S., Stefanie Schramm, Miriam C. Wolters, Julia Schmetzer, Christine V Möser, Claudia Knothe, Eduard Resch, Johannes Peil, Gerd Geisslinger, and Ellen Niederberger. 2016. “The Impact of Endurance Exercise on Global and AMPK Gene-Specific DNA Methylation.” *Biochemical and Biophysical Research Communications* 474(2):284–90.
Kobayashi, Yuya, Devin M. Absher, Zulfiqar G. Gulzar, Sarah R. Young, Jesse K. McKenney, Donna M. Peehl, James D. Brooks, Richard M. Myers, and Gavin Sherlock. 2011. “DNA Methylation Profiling Reveals Novel Biomarkers and

Important Roles for DNA Methyltransferases in Prostate Cancer." *Genome Research* 21(7):1017–27.
Korah, J., N. Falah, A. Lacerte, and J. J. Lebrun. 2012. "A Transcriptionally Active PRb-E2F1-P/CAF Signaling Pathway Is Central to TGFβ-Mediated Apoptosis." *Cell Death and Disease* 3(10):1–11.
Kruglyak, L. and D. a Nickerson. 2001. "Variation Is the Spice of Life." *Nature Genetics* 27(3):234–36.
Kuang, Jujiao, Xu Yan, Amanda J. Genders, Cesare Granata, and David J. Bishop. 2018. "An Overview of Technical Considerations When Using Quantitative Real-Time PCR Analysis of Gene Expression in Human Exercise Research." *PLoS ONE* 13(5):1–27.
Kuo, Kenneth C., Roy A. Mccune, Charles W. Gehrke, Rosemarie Midgett, and Melanie Ehrlich. 1980. "Quantitative Reversed-Phase High Performance Liquid Chromatographic Determination of Major and Modified Deoxyribonucleosides in DNA." *Nucleic Acids Research* 8(20):4763–76.
Kurdyukov, Sergey and Martyn Bullock. 2016. "DNA Methylation Analysis: Choosing the Right Method." *Biology* 5(1):3.
de la Rocha, Carmen, J. Eduardo Pérez-Mojica, Silvia Zenteno-De León, Braulio Cervantes-Paz, Fabiola E. Tristán-Flores, Dalia Rodríguez-Ríos, Jorge Molina-Torres, Enrique Ramírez-Chávez, Yolanda Alvarado-Caudillo, F. Javier Carmona, Manel Esteller, Rosaura Hernández-Rivas, Katarzyna Wrobel, Kazimierz Wrobel, Silvio Zaina, and Gertrud Lund. 2016. "Associations between Whole Peripheral Blood Fatty Acids and DNA Methylation in Humans." *Scientific Reports* 6(1):25867.
Laiglesia, L. M., S. Lorente-Cebrián, P. L. Prieto-Hontoria, M. Fernández-Galilea, S. M. R. Ribeiro, N. Sáinz, J. A. Martínez, and M. J. Moreno-Aliaga. 2016. "Eicosapentaenoic Acid Promotes Mitochondrial Biogenesis and Beige-like Features in Subcutaneous Adipocytes from Overweight Subjects." *The Journal of Nutritional Biochemistry* 37:76–82.
Lane, Stephen C., Donny M. Camera, David Gray Lassiter, José L. Areta, Stephen R. Bird, Wee Kian Yeo, Nikki A. Jeacocke, Anna Krook, Juleen R. Zierath, Louise M. Burke, and John A. Hawley. 2015. "Effects of Sleeping with Reduced Carbohydrate Availability on Acute Training Responses." *Journal of Applied Physiology* 119(6):643–55.
Laye, Matthew J. and Bente Klarlund Pedersen. 2010. "Acute Exercise and Ca2+ Stimulation Regulate Enzymes Involved in DNA Methylation in Human Skeletal Muscle." *Medicine & Science in Sports & Exercise* 42:23.
Lee, Ho-Sun, Albino Barraza-Villarreal, Hector Hernandez-Vargas, Peter D. Sly, Carine Biessy, Usha Ramakrishnan, Isabelle Romieu, and Zdenko Herceg. 2013. "Modulation of DNA Methylation States and Infant Immune System by Dietary Supplementation with ω-3 PUFA during Pregnancy in an Intervention Study." *The American Journal of Clinical Nutrition* 98(2):480–87.
Lee, I. Min, Eric J. Shiroma, Felipe Lobelo, Pekka Puska, Steven N. Blair, Peter T. Katzmarzyk, and Lancet Physical Activity Series Working Group. 2012. "Effect of Physical Inactivity on Major Non-Communicable Diseases Worldwide: An Analysis of Burden of Disease and Life Expectancy." *Lancet (London, England)* 380(9838):219–29.
Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. "The C. Elegans Heterochronic Gene Lin-4 Encodes Small RNAs with Antisense Complementarity to Lin-14." *Cell* 75(5):843–54.

Li, Jian, R. Alan Harris, Sau Wai Cheung, Cristian Coarfa, Mira Jeong, Margaret A. Goodell, Lisa D. White, Ankita Patel, Sung Hae Kang, Chad Shaw, A. Craig Chinault, Tomasz Gambin, Anna Gambin, James R. Lupski, and Aleksandar Milosavljevic. 2012. “Genomic Hypomethylation in the Human Germline Associates with Selective Structural Mutability in the Human Genome.” *PLoS Genetics* 8(5).

Li, Kelei, Tao Huang, Jusheng Zheng, Kejian Wu, and Duo Li. 2014. “Effect of Marine-Derived n-3 Polyunsaturated Fatty Acids on C-Reactive Protein, Interleukin 6 and Tumor Necrosis Factor α: A Meta-Analysis.” *PLoS ONE* 9(2):1–28.

Li, Yi-Ping. 2013. “TNF-α Is a Mitogen in Skeletal Muscle.” *American Journal of Physiology-Cell Physiology* 285(2):C370–76.

Lind, M. V., D. Martino, L. B. S. Harsl??f, Z. O. Kyjovska, M. Kristensen, and L. Lauritzen. 2015. “Genome-Wide Identification of Mononuclear Cell DNA Methylation Sites Potentially Affected by Fish Oil Supplementation in Young Infants: A Pilot Study.” *Prostaglandins Leukotrienes and Essential Fatty Acids* 101:1–7.

Lindholm, Maléne E., Francesco Marabita, David Gomez-Cabrero, Helene Rundqvist, Tomas J. Ekström, Jesper Tegnér, and Carl Johan Sundberg. 2015. “An Integrative Analysis Reveals Coordinated Reprogramming of the Epigenome and the Transcriptome in Human Skeletal Muscle after Training.” *Epigenetics* 9(12):1557–69.

Lisanti, Sofia, Wan A. W. Omar, Bartłomiej Tomaszewski, Sofie De Prins, Griet Jacobs, Gudrun Koppen, John C. Mathers, and Sabine A. S. Langie. 2013. “Comparison of Methods for Quantification of Global DNA Methylation in Human Cells and Tissues.” *PLoS ONE* 8(11):e79044.

Liu, C. C., T. T. Ou, C. C. Wu, R. N. Li, Y. C. Lin, C. H. Lin, W. C. Tsai, H. W. Liu, and J. H. Yen. 2011. “Global DNA Methylation, DNMT1, and MBD2 in Patients with Systemic Lupus Erythematosus.” *Lupus* 20(2):131–36.

Livak, Kenneth J. and Thomas D. Schmittgen. 2001. “Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method.” *Methods* 25(4):402–8.

Lochmann, Timothy L., Ravindar R. Thomas, James P. Bennett, and Shirley M. Taylor. 2015. “Epigenetic Modifications of the PGC-1α Promoter during Exercise Induced Expression in Mice.” *PLoS ONE* 10(6):1–16.

Lohner, Szimonetta, Katalin Fekete, Tamás Marosvölgyi, and Tamás Decsi. 2013. “Gender Differences in the Long-Chain Polyunsaturated Fatty Acid Status: Systematic Review of 51 Publications.” *Annals of Nutrition and Metabolism* 62(2):98–112.

Lu, Shan, Andrew P. Smith, Dan Moore, and Nancy M. Lee. 2010. “Different Real-Time PCR Systems Yield Different Gene Expression Values.” *Molecular and Cellular Probes* 24(5):315–20.

Lund, Jenny, Arild C. Rustan, Nils G. L??vsletten, Jonathan M. Mudry, Torgrim M. Langleite, Yuan Z. Feng, Camilla Stensrud, Mari G. Brubak, Christian A. Drevon, K??re I. Birkeland, Kristoffer J. Kolnes, Egil I. Johansen, Daniel S. Tangen, Hans K. Stadheim, Hanne L. Gulseth, Anna Krook, Eili T. Kase, J??rgen Jensen, and G. Hege Thoresen. 2017. “Exercise in Vivo Marks Human Myotubes in Vitro: Training-Induced Increase in Lipid Metabolism.” *PLoS ONE* 12(4):1–24.

Luo, Yanting, Xuemei Lu, and Hehuang Xie. 2014. “Dynamic Alu Methylation during

Normal Development, Aging, and Tumorigenesis." *BioMed Research International* 2014:1–12.
Ma, Yiyi, Caren E. Smith, Chao-qiang Lai, Marguerite R. Irvin, Laurence D. Parnell, Yu-chi Lee, Lucia D. Pham, Stella Aslibekyan, Steven A. Claas, Michael Y. Tsai, Ingrid B. Borecki, Edmond K. Kabagambe, José M. Ordovás, Devin M. Absher, and Donna K. Arnett. 2016. "The Effects of Omega-3 Polyunsaturated Fatty Acids and Genetic Variants on Methylation Levels of the Interleukin-6 Gene Promoter." *Molecular Nutrition & Food Research* 60(2):410–19.
MacIntyre, D. L., W. D. Reid, D. M. Lyster, I. J. Szasz, and D. C. McKenzie. 1996. "Presence of WBC, Decreased Strength, and Delayed Soreness in Muscle after Eccentric Exercise." *Journal of Applied Physiology (Bethesda, Md. : 1985)* 80(3):1006–13.
Mador, M. Jeffery, Erkan Bozkanat, Ajay Aggarwal, Mary Shaffer, and Thomas J. Kufel. 2004. "Endurance and Strength Training in Patients with COPD." *Chest* 125(6):2036–45.
Mahoney, D. J., A. Safdar, G. Parise, S. Melov, Minghua Fu, L. MacNeil, J. Kaczor, E. T. Payne, and M. A. Tarnopolsky. 2008. "Gene Expression Profiling in Human Skeletal Muscle during Recovery from Eccentric Exercise." *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 294(6):R1901-10.
Marques-Rocha, José Luiz, Fermin I. Milagro, Maria Luisa Mansego, Denise Machado Mourão, J. Alfredo Martínez, and Josefina Bressan. 2016. "LINE-1 Methylation Is Positively Associated with Healthier Lifestyle but Inversely Related to Body Fat Mass in Healthy Young Individuals." *Epigenetics* 11(1):49–60.
Marques, Camila Garcia, Vinicius Coneglian Santos, Adriana Cristina Levada-Pires, Thiago Manzoni Jacintho, Renata Gorjão, Tânia Cristina Pithon-Curi, and Maria Fernanda Cury-Boaventura. 2015. "Effects of DHA-Rich Fish Oil Supplementation on the Lipid Profile, Markers of Muscle Damage, and Neutrophil Function in Wheelchair Basketball Athletes before and after Acute Exercise." *Applied Physiology, Nutrition, and Metabolism* 40(6):596–604.
Martin, Elizabeth M. and Rebecca C. Fry. 2018. "Environmental Influences on the Epigenome: Exposure- Associated DNA Methylation in Human Populations." *Ssrn*.
Martorell, Miquel, Xavier Capó, Antoni Sureda, Joan M. Batle, Isabel Llompart, Emma Argelich, Josep A. Tur, and Antoni Pons. 2014. "Effect of DHA on Plasma Fatty Acid Availability and Oxidative Stress during Training Season and Football Exercise." *Food & Function* 5(8):1920–31.
Maston, Glenn A., Sara K. Evans, and Michael R. Green. 2006. "Transcriptional Regulatory Elements in the Human Genome." *Annual Review of Genomics and Human Genetics* 7(1):29–59.
Matelot, D., F. Schnell, G. Kervio, C. Ridard, N. Thillaye du Boullay, M. Wilson, and F. Carre. 2016. "Cardiovascular Benefits of Endurance Training in Seniors: 40 Is Not Too Late to Start." *International Journal of Sports Medicine* 37(08):625–32.
McGee, Sean L., David Sparling, Ann-Louise Olson, and Mark Hargreaves. 2006. "Exercise Increases MEF2- and GEF DNA-Binding Activity in Human Skeletal Muscle." *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 20(2):348–49.
McGhee, J. D. and G. Felsenfeld. 1980. "Nucleosome Structure." *Annual Review of Biochemistry* 49:1115–56.

McGlory, Chris, Stuart D. R. Galloway, D. Lee Hamilton, Craig McClintock, Leigh Breen, James R. Dick, John G. Bell, and Kevin D. Tipton. 2014. “Temporal Changes in Human Skeletal Muscle and Blood Lipid Composition with Fish Oil Supplementation.” *Prostaglandins Leukotrienes and Essential Fatty Acids* 90(6):199–206.

Metherel, A. H., J. M. Armstrong, A. C. Patterson, and K. D. Stark. 2009. “Assessment of Blood Measures of N-3 Polyunsaturated Fatty Acids with Acute Fish Oil Supplementation and Washout in Men and Women.” *Prostaglandins Leukotrienes and Essential Fatty Acids* 81(1):23–29.

Mickleborough, Timothy D., Jacob a Sinex, David Platt, Robert F. Chapman, and Molly Hirt. 2015. “The Effects PCSO-524®, a Patented Marine Oil Lipid and Omega-3 PUFA Blend Derived from the New Zealand Green Lipped Mussel (Perna Canaliculus), on Indirect Markers of Muscle Damage and Inflammation after Muscle Damaging Exercise in Untrained Men: A Random.” *Journal of the International Society of Sports Nutrition* 12(1):1–17.

Montaño, Alfonso, Marcos Hernández, Inmaculada Garrido, José Luís Llerena, and Francisco Espinosa. 2016. “Fatty Acid and Phenolic Compound Concentrations in Eight Different Monovarietal Virgin Olive Oils from Extremadura and the Relationship with Oxidative Stability.” *International Journal of Molecular Sciences* 17(11):1–20.

Moore, Lisa D., Thuc Le, and Guoping Fan. 2013. “DNA Methylation and Its Basic Function.” *Neuropsychopharmacology* 38(1):23–38.

Morlando, Mariangela and Alessandro Fatica. 2018. “Alteration of Epigenetic Regulation by Long Noncoding RNAs in Cancer.” *International Journal of Molecular Sciences* 19(2).

Mozaffarian, Dariush, Alberto Ascherio, Frank B. Hu, Meir J. Stampfer, Walter C. Willett, David S. Siscovick, and Eric B. Rimm. 2005. “Interplay Between Different Polyunsaturated Fatty Acids and Risk of Coronary Heart Disease in Men.” *Circulation* 111(2):157–64.

Muñoz-Cánoves, Pura, Camilla Scheele, Bente K. Pedersen, and Antonio L. Serrano. 2013. “Interleukin-6 Myokine Signaling in Skeletal Muscle: A Double-Edged Sword?” *FEBS Journal* 280(17):4131–48.

Murphy, Susan K., Zhiqing Huang, and Cathrine Hoyo. 2012. “Differentially Methylated Regions of Imprinted Genes in Prenatal, Perinatal and Postnatal Human Tissues.” *PLoS ONE* 7(7).

Na, Yeon Kyung, Hae Sook Hong, Won Kee Lee, Young Hun Kim, and Dong Sun Kim. 2015. “Increased Methylation of Interleukin 6 Gene Is Associated with Obesity in Korean Women.” *Molecules and Cells* 38(5):452–56.

Nakajima, K., M. Takeoka, M. Mori, S. Hashimoto, a Sakurai, H. Nose, K. Higuchi, N. Itano, M. Shiohara, T. Oh, and S. Taniguchi. 2010. “Exercise E Ff Ects on Methylation of ASC Gene.” 671–75.

Natale, Valéria Maria, Ingrid Karen Brenner, Andrei Ion Moldoveanu, Paris Vasiliou, Pang Shek, and Roy Jesse Shephard. 2003. “Effects of Three Different Types of Exercise on Blood Leukocyte Count during and Following Exercise.” *Sao Paulo Medical Journal = Revista Paulista de Medicina* 121(1):9–14.

Nicholas, Jennifer, Joachim G. Voss, Joyce Tsuji, Nadia D. Fulkerson, Julia Soulakova, and Barbara St Pierre Schneider. 2015. “Time Course of Chemokine Expression and Leukocyte Infiltration after Acute Skeletal Muscle Injury in Mice.” *Innate Immunity* 21(3):266–74.

Niculescu, Mihai D., Daniel S. Lupu, and Corneliu N. Craciunescu. 2013. “Perinatal

Manipulation of α-Linolenic Acid Intake Induces Epigenetic Changes in Maternal and Offspring Livers." *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 27(1):350–58.
Niculescu, Mihai D., Daniel S. Lupu, and Corneliu N. Craciunescu. 2014. "Alpha-Linolenic Acid Alters Cell Cycle, Apoptosis, and DNA Methyltransferase Expression in Mouse Neural Stem Cells, but Not Global DNA Methylation." *Journal of Human Nutrition and Food Science* 2(1):1026.
Nieman, David C., Dru A. Henson, Steven R. McAnulty, Fuxia Jin, and Kendra R. Maxwell. 2009. "N-3 Polyunsaturated Fatty Acids Do Not Alter Immune and Inflammation Measures in Endurance Athletes." *International Journal of Sport Nutrition and Exercise Metabolism* 19(5):536–46.
Nile, Christoper J., Robert C. Read, Mohammed Akil, Gordon W. Duff, and Anthony G. Wilson. 2008. "Methylation Status of a Single CpG Site in the IL6 Promoter Is Related to IL6 Messenger RNA Levels and Rheumatoid Arthritis." *Arthritis and Rheumatism* 58(9):2686–93.
Nitert, Marloes Dekker, Tasnim Dayeh, Peter Volkov, Targ Elgzyri, Elin Hall, Emma Nilsson, Beatrice T. Yang, Stefan Lang, Hemang Parikh, Ylva Wessman, Holger Weishaupt, Joanne Attema, Mia Abels, Nils Wierup, Peter Almgren, Per-Anders Jansson, Tina Rönn, Ola Hansson, Karl-Fredrik Eriksson, Leif Groop, and Charlotte Ling. 2012. "Impact of an Exercise Intervention on DNA Methylation in Skeletal Muscle from First-Degree Relatives of Patients with Type 2 Diabetes." *Diabetes* 61(12):3322–32.
Noehammer, Christa, Walter Pulverer, Melanie R. Hassler, Manuela Hofner, Matthias Wielscher, Klemens Vierlinger, Triantafillos Liloglou, David Mccarthy, Taylor J. Jensen, Anders Nygren, Henning Gohlke, Geert Trooskens, Maarten Braspenning, Wim Van Criekinge, Gerda Egger, and Andreas Weinhaeusel. 2014. "Strategies for Validation and Testing of DNA Methylation Biomarkers." *Epigenomics* 6(6):603–22.
Oberbach, Andreas, Stefanie Lehmann, Katharina Kirsch, Joanna Krist, Melanie Sonnabend, Axel Linke, Anke Tönjes, Michael Stumvoll, Matthias Blüher, and Peter Kovacs. 2008. "Long-Term Exercise Training Decreases Interleukin-6 (IL-6) Serum Levels in Subjects with Impaired Glucose Tolerance: Effect of the -174G/C Variant in IL-6 Gene." *European Journal of Endocrinology* 159(2):129–36.
Odom, Lawrence N. and Hugh S. Taylor. 2010. "Environmental Induction of the Fetal Epigenome." *Expert Review of Obstetrics & Gynecology* 5(6):657–64.
Ohno, S. 1972. "So Much 'Junk' DNA in Our Genome." *Brookhaven Symposia in Biology* 23:366–70.
Okano, Masaki, Daphne W. Bell, Daniel A. Haber, and En Li. 1999. "DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for de Novo Methylation and Mammalian Development." *Cell* 99(3):247–57.
Olins, Donald E. and Ada L. Olins. 2003. "Chromatin History: Our View from the Bridge." *Nature Reviews Molecular Cell Biology* 4(10):809–14.
Olson, Melissa V, Ying-Chun Liu, Bindi Dangi, J. Paul Zimmer, Norman Salem, and Julie M. Nauroth. 2013. "Docosahexaenoic Acid Reduces Inflammation and Joint Destruction in Mice with Collagen-Induced Arthritis." *Inflammation Research* 62(12):1003–13.
Ottman, Ruth. 1996. "Gene-Environment Interaction: Definitions and Study Designs." *Preventive Medicine* 25(6):764–70.
Paulsen, Gøran, Regina Crameri, Haakon Breien Benestad, Jan Gunnar Fjeld, Lars

Mørkrid, Jostein Hallén, and Truls Raastad. 2010. “Time Course of Leukocyte Accumulation in Human Muscle after Eccentric Exercise.” *Medicine & Science in Sports & Exercise* 42(1):75–85.
Peake, Jonathan, Kazunori Nosaka, and Katsuhiko Suzuki. 2005. “Characterization of Inflammatory Responses to Eccentric Exercise in Humans.” *Exercise Immunology Review* 11:64–85.
Pedersen, Bente K. and Mark A. Febbraio. 2008. “Muscle as an Endocrine Organ: Focus on Muscle-Derived Interleukin-6.” *Physiological Reviews* 88(4):1379–1406.
Peoples, Gregory E., Peter L. McLennan, Peter R. C. Howe, and Herbert Groeller. 2008. “Fish Oil Reduces Heart Rate and Oxygen Consumption during Exercise.” *Journal of Cardiovascular Pharmacology* 52(6):540–47.
Perfilyev, Alexander, Ingrid Dahlman, Linn Gillberg, Fredrik Rosqvist, David Iggman, Petr Volkov, Emma Nilsson, Ulf Risérus, and Charlotte Ling. 2017. “Impact of Polyunsaturated and Saturated Fat Overfeeding on the DNA-Methylation Pattern in Human Adipose Tissue: A Randomized Controlled Trial.” *American Journal of Clinical Nutrition* 105(4):991–1000.
Peschansky, Veronica J. and Claes Wahlestedt. 2014. “Non-Coding RNAs as Direct and Indirect Modulators of Epigenetic Regulation.” *Epigenetics* 9(1):3–12.
Petersen, A. M. W. and B. K. Pedersen. 2006. “The Role of IL-6 in Mediating the Anti-Inflammatory Effects of Exercise.” *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society* 57 Suppl 1(1):43–51.
Philpott, Jordan D., Oliver C. Witard, and Stuart D. R. Galloway. 2018. “Applications of Omega-3 Polyunsaturated Fatty Acid Supplementation for Sport Performance.” *Research in Sports Medicine* 00(00):1–19.
Plourde, Mélanie and Stephen C. Cunnane. 2007. “Extremely Limited Synthesis of Long Chain Polyunsaturates in Adults: Implications for Their Dietary Essentiality and Use as Supplements.” *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition et Metabolisme* 32(4):619–34.
Ponting, Chris P., Peter L. Oliver, and Wolf Reik. 2009. “Evolution and Functions of Long Noncoding RNAs.” *Cell* 136(4):629–41.
Popov, Daniil V., Evgeny A. Lysenko, Tatiana F. Vepkhvadze, Nadia S. Kurochkina, Pavel A. Maknovskii, and Olga L. Vinogradova. 2015. “Promoter-Specific Regulation of PPARGC1A Gene Expression in Human Skeletal Muscle.” *Journal of Molecular Endocrinology* 55(2):159–68.
Powers, Scott K., W. Bradley Nelson, and Matthew B. Hudson. 2011. “Exercise-Induced Oxidative Stress in Humans: Cause and Consequences.” *Free Radical Biology and Medicine* 51(5):942–50.
Pradhan, Sriharsa, Albino Bacolla, Robert D. Wells, and Richard J. Roberts. 1999. “Recombinant Human DNA (Cytosine-5) Methyltransferase.” *Journal of Biological Chemistry* 274(46):33002–10.
Proske, U. and D. L. Morgan. 2001. “Muscle Damage from Eccentric Exercise: Mechanism, Mechanical Signs, Adaptation and Clinical Applications.” *The Journal of Physiology* 537(Pt 2):333–45.
Quentin, Thomas, Julia Kitz, Michael Steinmetz, Andrea Poppe, Karin Bär, and Ralph Krätzner. 2011. “Different Expression of the Catalytic Alpha Subunits of the AMP Activated Protein Kinase--an Immunohistochemical Study in Human Tissue.” *Histology and Histopathology* 26(5):589–96.
Raastad, Truls, Jan Gunnar Fjeld, Jostein Hallén, Haakon Breien Benestad, and

Bjørn Audun Risøy. 2015. “Temporal Relation between Leukocyte Accumulation in Muscles and Halted Recovery 10–20 h after Strength Exercise.” *Journal of Applied Physiology* 95(6):2503–9.
Rahbar, Elaheh, Charlotte Mae K. Waits, Edward H. Kirby, Leslie R. Miller, Hannah C. Ainsworth, Tao Cui, Susan Sergeant, Timothy D. Howard, Carl D. Langefeld, and Floyd H. Chilton. 2018. “Allele-Specific Methylation in the FADS Genomic Region in DNA from Human Saliva, CD4+ Cells, and Total Leukocytes.” *Clinical Epigenetics* 10(1):46.
Ratnayake, W. M. Nimal and Claudio Galli. 2009. “Fat and Fatty Acid Terminology, Methods of Analysis and Fat Digestion and Metabolism: A Background Review Paper.” *Annals of Nutrition and Metabolism* 55(1–3):8–43.
Reid, Michael B. and Yi Ping Li. 2001. “Tumor Necrosis Factor-α and Muscle Wasting: A Cellular Perspective.” *Respiratory Research* 2(5):269–72.
Reinius, Lovisa E., Nathalie Acevedo, Maaike Joerink, Göran Pershagen, Sven Erik Dahlén, Dario Greco, Cilla Söderhäll, Annika Scheynius, and Juha Kere. 2012. “Differential DNA Methylation in Purified Human Blood Cells: Implications for Cell Lineage and Studies on Disease Susceptibility.” *PLoS ONE* 7(7).
Richard, C., E. D. Lewis, S. Goruk, and C. J. Field. 2016. “A Dietary Supply of Docosahexaenoic Acid Early in Life Is Essential for Immune Development and the Establishment of Oral Tolerance in Female Rat Offspring.” *Journal of Nutrition* 146(11):2398–2406.
Ririe, Kirk M., Randy P. Rasmussen, and Carl T. Wittwer. 1997. “Product Differentiation by Analysis of DNA Melting Curves during the Polymerase Chain Reaction.” *Analytical Biochemistry* 245(2):154–60.
Rivera, Chloe M. and Bing Ren. 2013. “Mapping Human Epigenomes.” *Cell* 155(1):39–55.
Roadmap Epigenomics Consortium, Anshul Kundaje, Wouter Meuleman, Jason Ernst, Misha Bilenky, Angela Yen, Alireza Heravi-Moussavi, Pouya Kheradpour, Zhizhuo Zhang, Jianrong Wang, Michael J. Ziller, Viren Amin, John W. Whitaker, Matthew D. Schultz, Lucas D. Ward, Abhishek Sarkar, Gerald Quon, Richard S. Sandstrom, Matthew L. Eaton, Yi-chieh Wu, Andreas R. Pfenning, Xinchen Wang, Melina Claussnitzer, Yaping Liu, Cristian Coarfa, R. Alan Harris, Noam Shoresh, Charles B. Epstein, Elizabeta Gjoneska, Danny Leung, Wei Xie, R. David Hawkins, Ryan Lister, Chibo Hong, Philippe Gascard, Andrew J. Mungall, Richard Moore, Eric Chuah, Angela Tam, Theresa K. Canfield, R. Scott Hansen, Rajinder Kaul, Peter J. Sabo, Mukul S. Bansal, Annaick Carles, Jesse R. Dixon, Kai-how Farh, Soheil Feizi, Rosa Karlic, Ah-Ram Kim, Ashwinikumar Kulkarni, Daofeng Li, Rebecca Lowdon, GiNell Elliott, Tim R. Mercer, Shane J. Neph, Vitor Onuchic, Paz Polak, Nisha Rajagopal, Pradipta Ray, Richard C. Sallari, Kyle T. Siebenthall, Nicholas A. Sinnott-Armstrong, Michael Stevens, Robert E. Thurman, Jie Wu, Bo Zhang, Xin Zhou, Arthur E. Beaudet, Laurie A. Boyer, Philip L. De Jager, Peggy J. Farnham, Susan J. Fisher, David Haussler, Steven J. M. Jones, Wei Li, Marco A. Marra, Michael T. McManus, Shamil Sunyaev, James A. Thomson, Thea D. Tlsty, Li-Huei Tsai, Wei Wang, Robert A. Waterland, Michael Q. Zhang, Lisa H. Chadwick, Bradley E. Bernstein, Joseph F. Costello, Joseph R. Ecker, Martin Hirst, Alexander Meissner, Aleksandar Milosavljevic, Bing Ren, John A. Stamatoyannopoulos, Ting Wang, and Manolis Kellis. 2015. “Integrative Analysis of 111 Reference Human Epigenomes.” *Nature* 518(7539):317–30.
Roberts, Thomas C. 2014. “The MicroRNA Biology of the Mammalian Nucleus.”

Molecular Therapy - Nucleic Acids 3(August):1–8.
Robson-Ansley, P. J., A. Saini, C. Toms, L. Ansley, I. H. Walshe, M. A. Nimmo, and J. A. Curtin. 2014. “Dynamic Changes in Dna Methylation Status in Peripheral Blood Mononuclear Cells Following an Acute Bout of Exercise: Potential Impact of Exercise-Induced Elevations in Interleukin-6 Concentration.” *Journal of Biological Regulators and Homeostatic Agents* 28(3):407–17.
Rodacki, L. F., Gleber Pereira, Katya Naliwaiko, Isabela Coelho, and Daniele Pequito. 2012. “Fish-Oil Supplementation Enhances the Effects of Strength Training in Elderly Women.” *Journal of Clinical Nutrition* (3):428–36.
Roessler, Claudia, Kevin Kuhlmann, Christine Hellwing, Anja Leimert, and Julia Schumann. 2017. “Impact of Polyunsaturated Fatty Acids on MiRNA Profiles of Monocytes/Macrophages and Endothelial Cells-A Pilot Study.” *International Journal of Molecular Sciences* 18(2):1–24.
Rönn, Tina, Petr Volkov, Cajsa Davegårdh, Tasnim Dayeh, Elin Hall, Anders H. Olsson, Emma Nilsson, Åsa Tornberg, Marloes Dekker Nitert, Karl Fredrik Eriksson, Helena a. Jones, Leif Groop, and Charlotte Ling. 2013. “A Six Months Exercise Intervention Influences the Genome-Wide DNA Methylation Pattern in Human Adipose Tissue.” *PLoS Genetics* 9(6).
Rosignoli, Patrizia, Raffaela Fuccelli, Roberto Fabiani, Maurizio Servili, and Guido Morozzi. 2013. “Effect of Olive Oil Phenols on the Production of Inflammatory Mediators in Freshly Isolated Human Monocytes.” *The Journal of Nutritional Biochemistry* 24(8):1513–19.
Rosqvist, Fredrik, David Iggman, Joel Kullberg, Jonathan Cedernaes, Hans-Erik Johansson, Anders Larsson, Lars Johansson, Håkan Ahlström, Peter Arner, Ingrid Dahlman, and Ulf Risérus. 2014. “Overfeeding Polyunsaturated and Saturated Fat Causes Distinct Effects on Liver and Visceral Fat Accumulation in Humans.” *Diabetes* 63(7):2356–68.
Ross, Robert M., Jayasimha N. Murthy, Istvan D. Wollak, and Andrew S. Jackson. 2010. “The Six Minute Walk Test Accurately Estimates Mean Peak Oxygen Uptake.” *BMC Pulmonary Medicine* 10.
Rowlands, D. S., R. A. Page, W. R. Sukala, M. Giri, S. D. Ghimbovschi, I. Hayat, B. S. Cheema, I. Lys, M. Leikis, P. W. Sheard, S. J. Wakefield, B. Breier, Y. Hathout, K. Brown, R. Marathi, F. E. Orkunoglu-Suer, J. M. Devaney, B. Leiken, G. Many, J. Krebs, W. G. Hopkins, and E. P. Hoffman. 2014. “Multi-Omic Integrated Networks Connect DNA Methylation and MiRNA with Skeletal Muscle Plasticity to Chronic Exercise in Type 2 Diabetic Obesity.” *Physiol Genomics* 46(20):747–65.
Ruas, Jorge L., Brian A. Irving, K. Sreekumaran Nair, Rajesh R. Rao, Jun Wu, Nicholas P. Greene, Jennifer L. Estall, Ian R. Lanza, James P. White, Brooke C. Harrison, Sandra Kleiner, Leslie A. Leinwand, Zhen Yan, Kyle A. Rasbach, Kevin T. Brannan, Mitsuharu Okutsu, and Bruce M. Spiegelman. 2012. “A PGC-1α Isoform Induced by Resistance Training Regulates Skeletal Muscle Hypertrophy.” *Cell* 151(6):1319–31.
Rudkowska, Iwona, Ann-Marie Paradis, Elisabeth Thifault, Pierre Julien, André Tchernof, Patrick Couture, Simone Lemieux, Olivier Barbier, and Marie-Claude Vohl. 2013. “Transcriptomic and Metabolomic Signatures of an N-3 Polyunsaturated Fatty Acids Supplementation in a Normolipidemic/Normocholesterolemic Caucasian Population.” *The Journal of Nutritional Biochemistry* 24(1):54–61.
Rudkowska, Iwona, Catherine Raymond, André Ponton, Hélène Jacques, Charles

Lavigne, Bruce J. Holub, André Marette, and Marie-Claude Vohl. 2011. "Validation of the Use of Peripheral Blood Mononuclear Cells as Surrogate Model for Skeletal Muscle Tissue in Nutrigenomic Studies." *OMICS: A Journal of Integrative Biology* 15(1–2):1–7.
Rundblad, Amanda, Kirsten B. Holven, Inge Bruheim, Mari C. Myhrstad, and Stine M. Ulven. 2018. "Effects of Fish and Krill Oil on Gene Expression in Peripheral Blood Mononuclear Cells and Circulating Markers of Inflammation: A Randomised Controlled Trial." *Journal of Nutritional Science* 7(e10).
Russo, Gian Luigi. 2009. "Dietary n - 6 and n - 3 Polyunsaturated Fatty Acids: From Biochemistry to Clinical Implications in Cardiovascular Prevention." *Biochemical Pharmacology* 77(6):937–46.
Rustan, Arild C. and Christian A. Drevon. 2005. "Fatty Acids: Structures and Properties." Pp. 1–7 in *Encyclopedia of Life Sciences*. Chichester: John Wiley & Sons, Ltd.
Saini, Amarjit, Adam P. Sharples, Nasser Al-Shanti, and Claire E. Stewart. 2017. "Omega-3 Fatty Acid EPA Improves Regenerative Capacity of Mouse Skeletal Muscle Cells Exposed to Saturated Fat and Inflammation." *Biogerontology* 18(1):109–29.
Saxonov, S., P. Berg, and D. L. Brutlag. 2006. "A Genome-Wide Analysis of CpG Dinucleotides in the Human Genome Distinguishes Two Distinct Classes of Promoters." *Proceedings of the National Academy of Sciences* 103(5):1412–17.
Scheller, Jürgen, Athena Chalaris, Dirk Schmidt-Arras, and Stefan Rose-John. 2011. "The Pro- and Anti-Inflammatory Properties of the Cytokine Interleukin-6." *Biochimica et Biophysica Acta - Molecular Cell Research* 1813(5):878–88.
Schnyder, Svenia and Christoph Handschin. 2015. "Skeletal Muscle as an Endocrine Organ: PGC-1α, Myokines and Exercise." *Bone* 80(1):115–25.
Seaborne, Robert A., Juliette Strauss, Matthew Cocks, Sam Shepherd, Thomas D. O'Brien, Ken A. Van Someren, Phillip G. Bell, Christopher Murgatroyd, James P. Morton, Claire E. Stewart, and Adam P. Sharples. 2018. "Human Skeletal Muscle Possesses an Epigenetic Memory of Hypertrophy." *Scientific Reports* 8(1):1–17.
Searles Nielsen, Susan, Harvey Checkoway, Rondi A. Butler, Heather H. Nelson, Federico M. Farin, W. T. Longstreth, Gary M. Franklin, Phillip D. Swanson, and Karl T. Kelsey. 2012. "LINE-1 DNA Methylation, Smoking and Risk of Parkinson's Disease." *Journal of Parkinson's Disease* 2(4):303–8.
Sharples, Adam P., Nasser Al-Shanti, and Claire E. Stewart. 2010. "C2 and C2C12 Murine Skeletal Myoblast Models of Atrophic and Hypertrophic Potential: Relevance to Disease and Ageing?" *Journal of Cellular Physiology* 225(1):240–50.
Sharples, Adam P., Ioanna Polydorou, David C. Hughes, Daniel J. Owens, Thomas M. Hughes, and Claire E. Stewart. 2016. "Skeletal Muscle Cells Possess a 'Memory' of Acute Early Life TNF-α Exposure: Role of Epigenetic Adaptation." *Biogerontology* 17(3):603–17.
Shastry, Barkur S. 2009. "SNPs: Impact on Gene Function and Phenotype." Pp. 3–22 in. Humana Press, Totowa, NJ.
Shaw, B., W. C. Leung, H. S. Tapp, A. L. Fitzpatrick, J. M. Saxton, and N. J. Belshaw. 2014. "A Change in Physical Activity Level Affects Leukocyte DNA Methylation of Genes Implicated in Cardiovascular Disease in the Elderly." *Proc. Physiol. Soc.* 31:C46.
Shukla, Sanjeev, Ersen Kavak, Melissa Gregory, Masahiko Imashimizu, Bojan

Shutinoski, Mikhail Kashlev, Philipp Oberdoerffer, Rickard Sandberg, and Shalini Oberdoerffer. 2011. “CTCF-Promoted RNA Polymerase II Pausing Links DNA Methylation to Splicing.” *Nature* 479(7371):74–79.

Sigal, Ronald J., Glen P. Kenny, Normand G. Boulé, George A. Wells, Denis Prud’homme, Michelle Fortier, Robert D. Reid, Heather Tulloch, Douglas Coyle, Penny Phillips, Alison Jennings, and James Jaffey. 2007. “Effects of Aerobic Training, Resistance Training, or Both on Glycemic Control in Type 2 Diabetes: A Randomized Trial.” *Annals of Internal Medicine* 147(6):357–69.

Silva, Gustavo J. J., Anja Bye, Hamid El Azzouzi, and Ulrik Wisløff. 2017. “MicroRNAs as Important Regulators of Exercise Adaptation.” *Progress in Cardiovascular Diseases* 60(1):130–51.

da Silva, Ivy Reichert Vital, Cintia Laura Pereira de Araujo, Gilson Pires Dorneles, Alessandra Peres, Andreia Luciana Bard, Gustavo Reinaldo, Paulo José Zimermann Teixeira, Pedro Dal Lago, and Viviane Rostirola Elsner. 2017. “Exercise-Modulated Epigenetic Markers and Inflammatory Response in COPD Individuals: A Pilot Study.” *Respiratory Physiology & Neurobiology* 242(March):89–95.

Silvennoinen, Mika, Juha P. Ahtiainen, Juha J. Hulmi, Satu Pekkala, Ritva S. Taipale, Bradley C. Nindl, Tanja Laine, Keijo Häkkinen, Harri Selänne, Heikki Kyröläinen, and Heikki Kainulainen. 2015. “PGC-1 Isoforms and Their Target Genes Are Expressed Differently in Human Skeletal Muscle Following Resistance and Endurance Exercise.” *Physiological Reports* 3(10):1–12.

Simar, David, Soetkin Versteyhe, Ida Donkin, Jia Liu, Luke Hesson, Vibe Nylander, Anna Fossum, and Romain Barrès. 2014. “DNA Methylation Is Altered in B and NK Lymphocytes in Obese and Type 2 Diabetic Human.” *Metabolism: Clinical and Experimental* 63(9):1188–97.

Simopoulos, Artemis P. 1999. “Essential Fatty Acids in Health and Chronic Disease.” *The American Journal of Clinical Nutrition* 70(3 Suppl):560S-569S.

Simopoulos, Artemis P. 2006. “Evolutionary Aspects of Diet, the Omega-6/Omega-3 Ratio and Genetic Variation: Nutritional Implications for Chronic Diseases.” *Biomedicine & Pharmacotherapy* 60(9):502–7.

Simopoulos, Artemis P. 2016. “An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity.” *Nutrients* 8(3):128.

Smith, Gordon I., Sophie Julliand, Dominic N. Reeds, David R. Sinacore, Samuel Klein, and Bettina Mittendorfer. 2015. “Fish Oil-Derived n-3 PUFA Therapy Increases Muscle Mass and Function in Healthy Older Adults.” *The American Journal of Clinical Nutrition* 102(1):115–22.

So, Mei Yu, Zhi Peng Tian, Yong Shian Phoon, Sha Sha, Michael N. Antoniou, Jiang Wen Zhang, Rudolf S. S. Wu, and Kian C. Tan-Un. 2014. “Gene Expression Profile and Toxic Effects in Human Bronchial Epithelial Cells Exposed to Zearalenone.” *PLoS ONE* 9(5).

Song, Liguo, Smitha R. James, Latif Kazim, and Adam R. Karpf. 2005. “Specific Method for the Determination of Genomic DNA Methylation by Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry.” *Analytical Chemistry* 77(2):504–10.

Spainhour, John CG, Hong Seo Lim, Soojin V Yi, and Peng Qiu. 2019. “Correlation Patterns Between DNA Methylation and Gene Expression in The Cancer Genome Atlas.” *Cancer Informatics* 18:117693511982877.

Steensberg, Adam, Christian P. Fischer, Charlotte Keller, Kirsten Møller, and Bente Klarlund Pedersen. 2003. “IL-6 Enhances Plasma IL-1ra, IL-10, and Cortisol in

Humans." *American Journal of Physiology - Endocrinology And Metabolism* 285(2):E433–37.
Steensberg, Adam, Gerrit van Hall, Takuya Osada, Massimo Sacchetti, Bengt Saltin, and B. Klarlund Pedersen. 2000. "Production of Interleukin-6 in Contracting Human Skeletal Muscles Can Account for the Exercise-Induced Increase in Plasma Interleukin-6." *The Journal of Physiology* 529 Pt 1(1):237–42.
Svec, David, Ales Tichopad, Vendula Novosadova, Michael W. Pfaffl, and Mikael Kubista. 2015. "How Good Is a PCR Efficiency Estimate: Recommendations for Precise and Robust QPCR Efficiency Assessments." *Biomolecular Detection and Quantification* 3:9–16.
Tachtsis, Bill, Donny Camera, and Orly Lacham-Kaplan. 2018. "Potential Roles of N-3 PUFAs during Skeletal Muscle Growth and Regeneration." *Nutrients* 10(3):1–20.
Tak, P. P. and G. S. Firestein. 2001. "NF-KappaB: A Key Role in Inflammatory Diseases." *The Journal of Clinical Investigation* 107(1):7–11.
Tani, Shigemasa, Atsuhiko Takahashi, Ken Nagao, and Atsushi Hirayama. 2015. "Association of Fish Consumption-Derived Ratio of Serum n-3 to n-6 Polyunsaturated Fatty Acids and Cardiovascular Risk With the Prevalence of Coronary Artery Disease." *International Heart Journal* 56(3):260–68.
Tartibian, Bakhtyar, Behzad Hajizadeh Maleki, and Asghar Abbasi. 2011. "11.11: Omega-3 Fatty Acids Supplementation Attenuates Inflammatory Markers after Eccentric Exercise in Untrained Men." *Clinical Journal of Sport Medicine : Official Journal of the Canadian Academy of Sport Medicine* 21(2):131–37.
Taverna, Sean D., Haitao Li, Alexander J. Ruthenburg, C. David Allis, and Dinshaw J. Patel. 2007. "How Chromatin-Binding Modules Interpret Histone Modifications: Lessons from Professional Pocket Pickers." *Nature Structural & Molecular Biology* 14(11):1025–40.
Tesena, Parichart, Wasamon Korchunjit, Jane Taylor, and Tuempong Wongtawan. 2017. "Comparison of Commercial RNA Extraction Kits and QPCR Master Mixes for Studying Gene Expression in Small Biopsy Tissue Samples from the Equine Gastric Epithelium." *Journal of Equine Science* 28(4):135–41.
Thomas, A. W., N. A. Davies, H. Moir, L. Watkeys, J. S. Ruffino, S. A. Isa, L. R. Butcher, M. G. Hughes, K. Morris, and R. Webb. 2012. "Exercise-Associated Generation of PPAR Ligands Activates PPAR Signaling Events and Upregulates Genes Related to Lipid Metabolism." *Journal of Applied Physiology* 112(5):806–15.
Tidball, James G. 2005. "Inflammatory Processes in Muscle Injury and Repair." *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 288(2):R345–53.
Tost, Jörg, Jenny Dunker, and Ivo Glynne Gut. 2003. "Analysis and Quantification of Multiple Methylation Variable Positions in CpG Islands by Pyrosequencing™." *BioTechniques* 35(1):152–56.
Tost, Jörg and Ivo G. Gut. 2007. "DNA Methylation Analysis by Pyrosequencing." *Nature Protocols* 2(9):2265–75.
Trebble, Timothy, Nigel K. Arden, Mike A. Stroud, Stephen A. Wootton, Graham C. Burdge, Elizabeth A. Miles, Anne B. Ballinger, Rachel L. Thompson, and Philip C. Calder. 2003. "Inhibition of Tumour Necrosis Factor-α and Interleukin 6 Production by Mononuclear Cells Following Dietary Fish-Oil Supplementation in Healthy Men and Response to Antioxidant Co-Supplementation." *British Journal of Nutrition* 90(02):405.

Tremblay, Bénédicte L., Frédéric Guénard, Iwona Rudkowska, Simone Lemieux, Patrick Couture, and Marie Claude Vohl. 2017. “Epigenetic Changes in Blood Leukocytes Following an Omega-3 Fatty Acid Supplementation.” *Clinical Epigenetics* 9(1):1–9.

Turner, Nigel, Clinton R. Bruce, Susan M. Beale, Kyle L. Hoehn, Trina So, Michael S. Rolph, and Gregory J. Cooney. 2007. “Excess Lipid Availability Increases Mitochondrial Fatty Acid Oxidative Capacity in Muscle: Evidence against a Role for Reduced Fatty Acid Oxidation in Lipid-Induced Insulin Resistance in Rodents.” *Diabetes* 56(8):2085–92.

Tyrrell, Daniel J., Manish S. Bharadwaj, Cynthia G. Van Horn, Stephen B. Kritchevsky, Barbara J. Nicklas, and Anthony J. A. Molina. 2015. “Respirometric Profiling of Muscle Mitochondria and Blood Cells Are Associated With Differences in Gait Speed Among Community-Dwelling Older Adults.” *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 70(11):1394–99.

Valasek, M. A. and J. J. Repa. 2005. “The Power of Real-Time PCR.” *Adv Physiol Educ* 29(3):151–59.

Varela-Moreiras, Gregorio, Emma Ruiz, Teresa Valero, José Manuel Avila, and Susana del Pozo. 2013. “The Spanish Diet: An Update.” *Nutrición Hospitalaria* 28 Suppl 5:13–20.

Vedin, Inger, Tommy Cederholm, Yvonne Freund-Levi, Hans Basun, Anita Garlind, Gerd Faxén Irving, Maria Eriksdotter-Jönhagen, Lars Olof Wahlund, Ingrid Dahlman, and Jan Palmblad. 2012. “Effects of DHA- Rich n-3 Fatty Acid Supplementation on Gene Expression in Blood Mononuclear Leukocytes: The OmegAD Study.” *PLoS ONE* 7(4):1–8.

Vedin, Inger, Tommy Cederholm, Yvonne Freund Levi, Hans Basun, Anita Garlind, Gerd Faxén Irving, Maria Eriksdotter Jönhagen, Bengt Vessby, Lars-Olof Wahlund, and Jan Palmblad. 2008. “Effects of Docosahexaenoic Acid-Rich n-3 Fatty Acid Supplementation on Cytokine Release from Blood Mononuclear Leukocytes: The OmegAD Study.” *The American Journal of Clinical Nutrition* 87(6):1616–22.

Ventura-Clapier, Renée, Anne Garnier, and Vladimir Veksler. 2008. “Transcriptional Control of Mitochondrial Biogenesis: The Central Role of PGC-1alpha.” *Cardiovascular Research* 79(2):208–17.

Verlengia, R., R. Gorjao, C. Kanunfre, S. Bordin, T. Martinsdelima, E. Martins, and R. Curi. 2004. “Comparative Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Proliferation, Cytokine Production, and Pleiotropic Gene Expression in Jurkat Cells.” *The Journal of Nutritional Biochemistry* 15(11):657–65.

Vermeulen, Joëlle, Katleen De Preter, Steve Lefever, Justine Nuytens, Fanny De Vloed, Stefaan Derveaux, Jan Hellemans, Frank Speleman, and Jo Vandesompele. 2011. “Measurable Impact of RNA Quality on Gene Expression Results from Quantitative PCR.” *Nucleic Acids Research* 39(9).

Visser, M., M. Pahor, D. R. Taaffe, B. H. Goodpaster, E. M. Simonsick, A. B. Newman, M. Nevitt, T. B. Harris, and A. Series. 2002. “Relationship of Interleukin-6 and Tumor Necrosis Factor-Alpha with Muscle Mass and Muscle Strength in Elderly Men and Women: The Health The Journals of Gerontology Biological Sciences and Medical Sciences, :.” 57(5 SRC-GoogleScholar FG-0):M326-32.

Voisin, Sarah, Markus S. Almén, George Moschonis, George P. Chrousos, Yannis

Manios, and Helgi B. Schiöth. 2014. “Dietary Fat Quality Impacts Genome-Wide DNA Methylation Patterns in a Cross-Sectional Study of Greek Preadolescents.” *European Journal of Human Genetics : EJHG* (January):1–9.
Waddington, C. H. 2012. “The Epigenotype. 1942.” *International Journal of Epidemiology* 41(1):10–13.
Walshe, I., P. Robson-Ansley, A. St Clair Gibson, C. Lawrence, K. G. Thompson, and L. Ansley. 2010. “The Reliability of the IL-6, SIL-6R and Sgp130 Response to a Preloaded Time Trial.” *European Journal of Applied Physiology* 110(3):619–25.
Warnecke, Peter M., Clare Stirzaker, John R. Melki, Douglas S. Millar, Cheryl L. Paul, and Susan J. Clark. 1997. “Detection and Measurement of PCR Bias in Quantitative Methylation Analysis of Bisulphite-Treated DNA.” *Nucleic Acids Research* 25(21):4422–26.
Watson, James and Francis Crick. 1953. “Molecular Structure of Nucleic Acids.” *Nature.* 171(4356):737–38.
Witczak, C. A., C. G. Sharoff, and L. J. Goodyear. 2008. “AMP-Activated Protein Kinase in Skeletal Muscle: From Structure and Localization to Its Role as a Master Regulator of Cellular Metabolism.” *Cellular and Molecular Life Sciences : CMLS* 65(23):3737–55.
Wojdacz, Tomasz K. and Alexander Dobrovic. 2007. “Methylation-Sensitive High Resolution Melting (MS-HRM): A New Approach for Sensitive and High-Throughput Assessment of Methylation.” *Nucleic Acids Research* 35(6):e41.
Wojdacz, Tomasz K., Lise Lotte Hansen, and Alexander Dobrovic. 2008. “A New Approach to Primer Design for the Control of PCR Bias in Methylation Studies.” *BMC Research Notes* 1:3–5.
Wong, Albert H. C., Irving I. Gottesman, and Arturas Petronis. 2005. “Phenotypic Differences in Genetically Identical Organisms: The Epigenetic Perspective.” *Human Molecular Genetics* 14 Spec No(SPEC. ISS. 1):R11-8.
Wong, Marisa L. and Juan F. Medrano. 2005. “Real-Time PCR for MRNA Quantitation.” *BioTechniques* 39(1):75–85.
World health organisation, WHO. 2010. “Global Recommendations on Physical Activity for Health.”
Wu, Hui-Chen, Lissette Delgado-Cruzata, Julie D. Flom, Maya Kappil, Jennifer S. Ferris, Yuyan Liao, Regina M. Santella, and Mary Beth Terry. 2011. “Global Methylation Profiles in DNA from Different Blood Cell Types.” *Epigenetics* 6(1):76–85.
Wu, Yan, Estil Strawn, Zainab Basir, Gloria Halverson, and Sun Wei Guo. 2007. “Aberrant Expression of Deoxyribonucleic Acid Methyltransferases DNMT1, DNMT3A, and DNMT3B in Women with Endometriosis.” *Fertility and Sterility* 87(1):24–32.
Xiao, Min, Jin Li, Wei Li, Yu Wang, Feizhen Wu, Yanping Xi, Lan Zhang, Chao Ding, Huaibing Luo, Yan Li, Lina Peng, Liping Zhao, Shaoliang Peng, Yao Xiao, Shihua Dong, Jie Cao, and Wenqiang Yu. 2017. “MicroRNAs Activate Gene Transcription Epigenetically as an Enhancer Trigger.” *RNA Biology* 14(10):1326–34.
Xiong, Zhenggang and Peter W. Laird. 1997. “COBRA: A Sensitive and Quantitative DNA Methylation Assay.” *Nucleic Acids Research* 25(12):2532–34.
Xu, Yurui, Lin Chao, Jianyu Wang, and Yonghong Sun. 2017. “MiRNA-148a Regulates the Expression of the Estrogen Receptor through DNMT1-Mediated DNA Methylation in Breast Cancer Cells.” *Oncology Letters* 14(4):4736–40.

Yakeu, G., L. Butcher, S. Isa, R. Webb, A. W. Roberts, A. W. Thomas, K. Backx, P. E. James, and K. Morris. 2010. “Low-Intensity Exercise Enhances Expression of Markers of Alternative Activation in Circulating Leukocytes: Roles of PPARγ and Th2 Cytokines.” *Atherosclerosis* 212(2):668–73.

Yaqoob, Parveen and Philip C. Calder. 1995. “Effects of Dietary Lipid Manipulation upon Inflammatory Mediator Production by Murine Macrophages.” *Cellular Immunology* 163(1):120–28.

Yarla, Nagendra S., Angela Polito, and Ilaria Peluso. 2017. “Effects of Olive Oil on TNF-α and IL-6 in Humans: Implication in Obesity and Frailty.” *Endocrine, Metabolic & Immune Disorders - Drug Targets* 18(1):63–74.

Ye, Jian, George Coulouris, Irena Zaretskaya, Ioana Cutcutache, Steve Rozen, and Thomas L. Madden. 2012. “Primer- BLAST: A Tool to Design Target- Specific Primers for Polymerase Chain Reaction.”

Zhang, Fangyuan and Degeng Wang. 2017. “The Pattern of MicroRNA Binding Site Distribution.” *Genes* 8(11).

Zhang, Jun, Cuizhe Wang, Xiaodan Ha, Wei Li, Peng Xu, Yajuan Gu, Tingting Wang, Yan Wang, and Jianxin Xie. 2017. “DNA Methylation of Tumor Necrosis Factor-α, Monocyte Chemoattractant Protein-1, and Adiponectin Genes in Visceral Adipose Tissue Is Related to Type 2 Diabetes in the Xinjiang Uygur Population.” *Journal of Diabetes* 9(7):699–706.

Zhang, Y., S. Hashimoto, C. Fujii, S. Hida, K. Ito, T. Matsumura, T. Sakaizawa, M. Morikawa, S. Masuki, H. Nose, K. Higuchi, K. Nakajima, and S. Taniguchi. 2015. “NFκB2 Gene as a Novel Candidate That Epigenetically Responds to Interval Walking Training.” *International Journal of Sports Medicine* 36(9):769–75.

Zierath, Juleen R. and John a Hawley. 2004. “Skeletal Muscle Fiber Type: Influence on Contractile and Metabolic Properties.” *PLoS Biology* 2(10):e348.

Appendices

Appendix A – Published manuscript

EPIGENETICS
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RESEARCH PAPER

Impact of aerobic exercise and fatty acid supplementation on global and gene-specific DNA methylation

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ABSTRACT

Lifestyle interventions, including exercise and dietary supplementation, can modify DNA methylation and exert health benefits; however, the underlying mechanisms are poorly understood. Here we investigated the impact of acute aerobic exercise and the supplementation of omega-3 polyunsaturated fatty acids (n-3 PUFA) and extra virgin olive oil (EVOO) on global and gene-specific (*PPARGC1A, IL6* and *TNF*) DNA methylation, and DNMT mRNA expression in leukocytes of disease-free individuals. Eight trained male cyclists completed an exercise test before and after a four-week supplementation of n-3 PUFA and EVOO in a double-blind, randomised, repeated measures design. Exercise triggered global hypomethylation (Pre 79.2%; Post 78.7%; $p = 0.008$), alongside, hypomethylation (Pre 6.9%; Post 6.3%; $p < 0.001$) and increased mRNA expression of PPARGC1A ($p < 0.001$). Associations between *PPARGC1A* methylation and exercise performance were also detected. An interaction between supplement and trial was detected for a single CpG of IL6 indicating increased DNA methylation following n-3 PUFA and decreased methylation following EVOO ($p = 0.038$). Global and gene-specific DNA methylation associated with markers of inflammation and oxidative stress. The supplementation of EVOO reduced DNMT1 mRNA expression compared to n-3 PUFA supplementation ($p = 0.048$), whereas, *DNMT3a* ($p = 0.018$) and *DNMT3b* ($p = 0.046$) mRNA expression were decreased following exercise. In conclusion, we demonstrate that acute exercise and dietary supplementation of n-3 PUFAs and EVOO induce DNA methylation changes in leukocytes, potentially via the modulation of *DNMT* mRNA expression. Future studies are required to further elucidate the impact of lifestyle interventions on DNA methylation.

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DNA methylation; exercis
inflammation; n-3 PUFA

Introduction

Environmental stimuli, including exercise and dietary interventions, can modify the DNA methylome at a global and gene-specific level [1]. Exercise training studies have demonstrated hypomethylation of the genome following exercise in both skeletal muscle [2–4] and blood leukocytes [5–7]. Within skeletal muscle, acute exercise has been demonstrated to induce hypomethylation [4,8–10]; however, the only investigation of DNA methylation in leukocytes following acute exercise failed to detect any changes in DNA methylation [11]. Despite the scarcity of literature surrounding the impact of acute exercise on DNA methylation in leukocytes, an epigenetic consequence is suggested by the remodelling of the leukocyte transcriptome [12–14].

Acute exercise is associated with adjustments in t expression of genes involved in a variety of cellul processes, including immune response mitochondri biogenesis, metabolism and muscle remodelling [1 16]. The *PPARGC1A* gene, which encodes for pero some proliferator-activated receptor gamma, c activator alpha (PGC1-a), is known as the mast regulator of mitochondrial biogenesis and plays important role in aerobic training adaptation [1 In immune cells, PPARGC1A is associated with an inflammatory [18,19] and anti-oxidant defence [2 however, the impact of exercise-induced inflamm tion and oxidative stress on PPARGC1A DNA meth lation is unknown. Epigenetic studies have link a CpG site −260 bases from the promoter *PPARCG1A* with the regulation of mRNA expressio In skeletal muscle, exercise can demethylate t

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Appendix B – Example informed consent form

Loughborough University

[Insert study title].
INFORMED CONSENT FORM
(to be completed after Participant Information Sheet has been read)

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.	Yes ☐	No ☐
I have read and understood the information sheet and this consent form.	Yes ☐	No ☐
I have had an opportunity to ask questions about my participation.	Yes ☐	No ☐
I understand that I am under no obligation to take part in the study.	Yes ☐	No ☐
I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.	Yes ☐	No ☐
I understand that all the 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.	Yes ☐	No ☐
I agree to participate in this study.	Yes ☐	No ☐
I agree that the bodily samples taken during this study can be stored for future research.	Yes ☐	No ☐
If No to above, I confirm that the bodily samples taken during this study can **only be** used for this study and should be disposed of after 5 years.	Yes ☐	No ☐

Your name _____

Your signature _____

Signature of investigator _____

Date _____

Appendix C – Health Screen Questionnaire

Loughborough University

Participant ID .....…….

Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

Please complete this brief questionnaire to confirm your fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a)	on medication, prescribed or otherwise	Yes		No		
(b)	attending your general practitioner	Yes		No		
(c)	on a hospital waiting list	Yes		No		

2. **In the past two years,** have you had any illness or injury which required you to:

(a)	consult your GP	Yes		No		
(b)	attend a hospital outpatient department.....	Yes		No		
(c)	be admitted to hospital	Yes		No		

3. **Have you ever** had any of the following:

(a)	Convulsions/epilepsy	Yes		No		
(b)	Asthma	Yes		No		
(c)	Eczema	Yes		No		
(d)	Diabetes	Yes		No		
(e)	A blood disorder	Yes		No		
(f)	Head injury	Yes		No		

(g)	Digestive problems	Yes		No	
(h)	Heart problems/chest pains ……	Yes		No	
(i)	Problems with muscles, bones or joints	Yes		No	
(j)	Disturbance of balance/coordination	Yes		No	
(k)	Numbness in hands or feet	Yes		No	
(l)	Disturbance of vision	Yes		No	
(m)	Ear/hearing problems	Yes		No	
(n)	Thyroid problems	Yes		No	
(o)	Kidney or liver problems	Yes		No	
(p)	Problems with blood pressure	Yes		No	

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....
.....

4. Smoking, physical activity and family history

(a)	Are you a current or recent (within the last six months) smoker?	Yes		No	
(b)	Are you physically active (30 minutes of moderate intensity, physical activity on at least 3 days each week for at least 3 months)?	Yes		No	
(c)	Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?	Yes		No	

5. Allergy Information

(a)	Are you allergic to any food products?	Yes		No	
(b)	Are you allergic to any medicines?	Yes		No	
(c)	Are you allergic to plasters?	Yes		No	
(d)	Are you allergic to latex?	Yes		No	

If YES to any of the above, please provide additional information on the allergy

.....

6. Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details.

……

7. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name:
……

Telephone Number:
……

Work ☐ Home ☐ Mobile ☐

Relationship to Participant:……

Appendix D – Physical Activity Questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

The questions are about the time you spent being physically active in the last 7 days. They include questions about activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Please answer each question even if you do not consider yourself to be an active person.

In answering the following questions,

- **vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder that normal.

- **moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder that normal.

1a. During the last 7 days, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling,?

Think about *only* those physical activities that you did for at least 10 minutes at a time.

_____ **days per week** ⇨

1b. How much time in total did you usually spend on one of those days doing vigorous physical activities?

_____ **hours** _____ **minutes**

or

☐ **none**

2a. Again, think *only* about those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ **days per week** ⇨

2b. How much time in total did you usually spend on one of those days doing moderate physical activities?

_____ **hours** _____ **minutes**

or

☐ **none**

3a. During the last 7 days, on how many days did you **walk** for at least 10 minutes at a time? This includes walking at work and at home, walking to travel from place to place and any other walking that you did solely for recreation, sport, exercise or leisure.

_____ **days per week** ⇨

3b. How much time in total did you usually spend walking on one of those days?

_____ **hours** _____ **minutes**

or

☐ **none**

The last question is about the time you spent sitting on weekdays while at work, at home, while doing course work and during leisure time. This includes time spent sitting at a desk, visiting friends, reading traveling on a bus or sitting or lying down to watch television.

4. During the last 7 days, how much time in total did you usually spend *sitting* on a **week day?**

____ **hours** _____ **minutes**

This is the end of questionnaire, thank you for participating.

This is the final SHORT LAST 7 DAYS SELF-ADMINISTERED version of IPAQ from the 2000/01 Reliability and Validity Study. Completed May 2001.

Appendix E – Dietary record

Dietary record sheet

Please fill out the food and drink as you consume it, include everything even water. Every individual food should have its own row. Please include as much information about the portion size, weights (please try to weigh all food), preparation method and brand as you can.

Time	Meal	Food / Drink	Brand	Preparation	Amount
E.g. 9.00	Breakfast	Bread	Hovis	Toasted	2 slices
		Butter	Lurpack	Refrigerated	10g

Appendix F – DNA Extraction protocols

i QIAamp DNA Blood Midi Protocol

1) Pipet 200 µL QIAGEN Protease into the bottom of a 15 mL centrifuge tube.
2) Add 2 mL blood and mix briefly.
3) Add 2.4 mL Buffer AL, and mix thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for at least 1 min.
4) Incubate at 70°C for 10 min.
5) Add 2 mL ethanol (96–100%) to the sample, and mix by inverting the tube 10 times, followed by additional vigorous shaking.
6) Carefully transfer one half of the solution from step 5 onto the QIAamp Midi column placed in a 15 mL centrifuge tube, taking care not to moisten the rim. Close the cap and centrifuge at 1850 x g for 3 min.

Note: If the solution has not completely passed through the membrane, centrifuge again at a slightly higher speed.

7) Remove the QIAamp Midi column, discard the filtrate, and place the QIAamp Midi column back into the 15 mL centrifuge tube. Load the remainder of the solution from step 5 onto the QIAamp Midi column. Close the cap and centrifuge again at 1850 x g for 3 min.
8) Remove the QIAamp Midi column, discard the filtrate, and place the QIAamp Midi column back into the 15 mL centrifuge tube.
9) Carefully, without moistening the rim, add 2 mL Buffer AW1 to the QIAamp Midi column. Close the cap and centrifuge at 4500 x g for 1 min.

Note: Do not discard the flow-through at this stage. Continue directly with step 10.

10) Carefully, without moistening the rim, add 2 mL Buffer AW2 to the QIAamp Midi column. Close the cap and centrifuge at 4500 x g for 15 min.
11) Place the QIAamp Midi column in a clean 15 mL centrifuge tube , and discard the collection tube containing the filtrate.
12) Pipet 300 µL Buffer AE directly onto the membrane of the QIAamp Midi column and close the cap. Incubate at room temperature for 5 min, and centrifuge at 4500 x g for 2 min.
13) Reload the eluate (300 µL) containing the DNA onto the membrane of the QIAamp Midi column. Close the cap and incubate at room temperature for 5 min. Centrifuge at 4500 x g for 2 min.
14) Discard the QIAamp Midi column, and save eluate.

ii ReliaPrep Blood gDNA Miniprep System

1) Ensure blood sample is fully thawed and mix the sample for 10 minutes on a rotisserie shaker at room temperature.
2) Add 20µL of Proteinase K into a 1.5mL microcentrifuge tube.
3) Add 200µL of blood to the tube and briefly mix.
4) Add 200µL of Cell Lysis Buffer to the tube. Mix by vortexing for at least 10 seconds.
5) Incubate at 56°C for 10 minutes.
6) Add 250µL of Binding Buffer to the tube and mix by vortexing for 10 seconds with a vortex mixer.

Note: The lysate should be dark green at this point.

7) Add the contents of the tube to a ReliaPrep Binding Column and place it in a microcentrifuge.
8) Centrifuge for 1 minute at maximum speed. Check the binding column to make sure the lysate has completely passed through the membrane. If lysate is still visible on top of the membrane, centrifuge the column for another minute.
9) Remove the collection tube and discard the flowthrough.
10) Place the binding column into a fresh collection tube. Add 500µL of Column Wash Solution to the column, and centrifuge for 3 minutes at maximum speed. Discard the flowthrough.

Note: If any of the wash solution remains on the membrane, centrifuge the column for another minute.

11) Repeat Step 11 twice for a total of three washes.
12) Place the column in a clean 1.5mL microcentrifuge tube.
13) Add 50µL of Nuclease-Free Water to the column. Centrifuge for 1 minute at maximum speed.
14) Discard the ReliaPrep Binding Column and save eluate.

Appendix G – Bisulfite Conversion protocol

i EpiTect Fast Bisulfite protocol

Part 1 - Bisulfite conversion protocol

1) Prepare the bisulfite reactions in 200 µL PCR tubes. Add each component in the order listed in the table below.

Reagent	Volume per sample
DNA*	20
Bisulfite solution	85
DNA protect buffer	35
Total	140

*Maximum of 2 µg of DNA to be added used nuclease free water for remaining volume

2) Close the PCR tubes and mix the bisulfite reactions thoroughly.
   Note: DNA Protect Buffer should turn from green to blue
3) Perform the bisulfite DNA conversion using the following cycling conditions

Stage	Time	Temperature
Denaturation	5 min	95°C
Incubation	20 min	60°C
Denaturation	5 min	95°C
Incubation	20 min	60°C
Hold	Indefinite	20°C

Part 2 - Cleanup of bisulfite-converted DNA

1) Briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 mL microcentrifuge tubes.

Note: Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

2) Add 310 µL freshly prepared Buffer BL to each sample. Mix the solutions by vortexing and then centrifuge briefly.
3) Add 250 µL ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 s, and centrifuge briefly to remove the drops from inside the lid.
4) Transfer the entire mixture from each tube from into the corresponding MinElute DNA spin column.

5) Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
6) Add 500 µL Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
7) Add 500 µL Buffer BD to each spin column, and incubate for 15 min at room temperature

Note: If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

8) Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
9) Add 500 µL Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
10) Repeat the previous step
11) Add 250 µL ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 min.
12) Place the spin columns into new 2 mL collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.
13) Place the spin columns with open lids into a clean 1.5 mL microcentrifuge tube and incubate the columns for 5 min at 60°C in a heating block.
14) Place the spin columns into clean 1.5 mL microcentrifuge tubes. Add 15 µL Buffer EB (elution buffer) directly onto the center of each spin-column membrane and close the lids gently.
15) Incubate the spin columns at room temperature for 1 min.
16) Centrifuge for 1 min at 15,000 x g to elute the DNA.

ii EpiTect LyseAll Lysis Protocol

Part 1a - Whole blood

1) Dilute 20 µL of whole blood sample with 380 µL Buffer EL
2) Incubate at room temperature for 15 min. Invert tubes during incubation.
3) Centrifuge at maximum speed for 5 min.
4) Discard supernatant and add an additional 125 µL Buffer EL.
5) Centrifuge at maximum speed for 1 min.
6) Resuspend pellet in 10 µL PBS and transfer into 8-well strips.
7) Add 10 µL distilled water, 15 µL Lysis Buffer FTB, and 5 µL proteinase K.
8) Continue with Part 2.

Part 1b - Skeletal muscle samples

1) Place the tissue (max 100 µg) into a 8-well strips.
2) Add 20 µL distilled water to the tissue sample
3) Add 15 µL Lysis Buffer FTB and 5 µL proteinase K.
4) Continue with Part 2.

Part 2

1) Vortex and briefly centrifuge the samples.
2) Incubate samples for 30 min at 56°C.
3) Proceed with EpiTect Fast Bisulfite protocol (**Appendix Gi**)

Appendix H – RNA extraction protocols

i Tempus Spin RNA Isolation protocol

Part 1 – Isolate RNA

1) Remove the cap from the Tempus™ Blood RNA Tube, then pour the contents into a clean 50-mL conical tube.
2) Add 3 mL of 1× PBS into the conical tube.
3) Vortex the tube vigorously (at maximum vortex speed) for 30 seconds.
4) Centrifuge the tube at 4°C at 3,000 × g for 30 minutes.
5) Carefully pour off the supernatant. Ensuring that you do not dislodge the RNA pellet.
6) Leave the conical tube inverted on absorbent paper for 1 to 2 minutes.
7) Blot the remaining drops of liquid off the rim of the conical tube with clean absorbent paper.
8) Add 400 µL of RNA Purification Resuspension Solution into the conical tube, then vortex briefly to resuspend the RNA pellet.

Part 2 – RNA purification

1) Pre-wet the filtration membrane by adding 100 µL of RNA Purification Wash Solution 1 into the purification filter.
2) Add ~400 µL of the resuspended RNA into the purification filter, then centrifuge for 30 seconds at 16,000 × g.
3) Remove the purification filter, discard the liquid waste collected in the waste tube, then re-insert the purification filter into the waste tube.
4) Add 500 µL of RNA Purification Wash Solution 1 into the purification filter, then centrifuge for 30 seconds at 16,000 × g.
5) Remove the purification filter, discard the liquid waste and re-insert the purification filter into the waste tube.
6) Add 500 µL of RNA Purification Wash Solution 2 into the purification filter, then centrifuge for 30 seconds at 16,000 × g.
7) Remove the purification filter, discard the liquid waste and re-insert the purification filter into the waste tube.
8) Repeat the RNA Purification Wash Solution 2 wash step
9) Centrifuge for 30 seconds at 16,000 × g to dry the membrane.
10) Transfer the purification filter to a new, labelled collection tube.
11) Add 100 µL Nucleic Acid Purification Elution Solution and incubate for 2 minutes at 70°C, then centrifuge for 30 seconds at 16,000 × g.
12) Add ~100 µL of the collected RNA eluate back into the purification filter, then centrifuge for 2 minutes at 16,000–18,000 × g. No incubation.
13) Transfer 90 µL of the RNA eluate to a new labelled collection tube.
14) Replace the cap on the new collection tube, then store the RNA at −20°C, or −80°C for long-term storage.

ii TRIzol LS / TRI Reagent protocol

Part 1 a – Whole blood samples

1) Add 0.75mL of TRIzol LS to 0.25mL of whole blood.
2) Homogenize by aspiration with pipette.

Part 1b – Skeletal muscle samples

1) Add 1mL of TRI Reagent into a 2mL tube containing skeletal muscle tissue.
2) Homogenize using TissueLyser II (Qiagen, Germany) and 5mm Stainless steel beads (Qiagen, Germany).

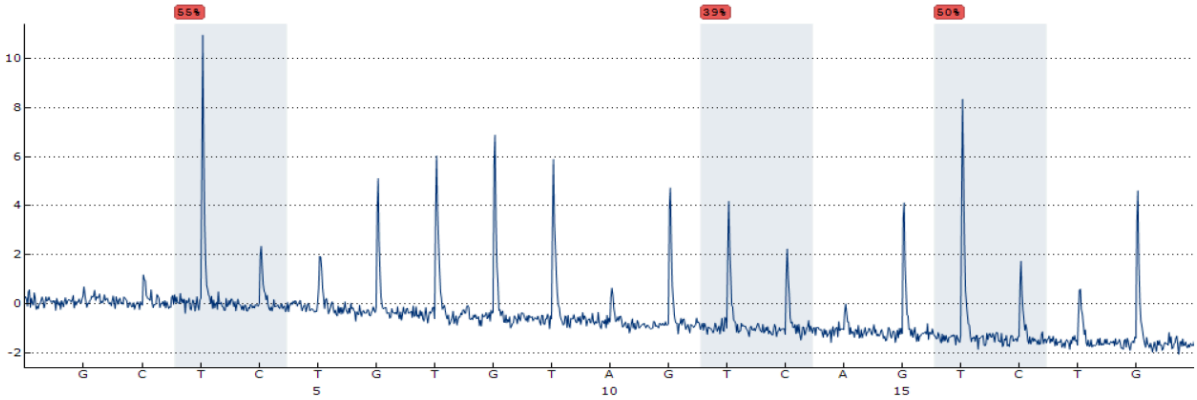
Part 2

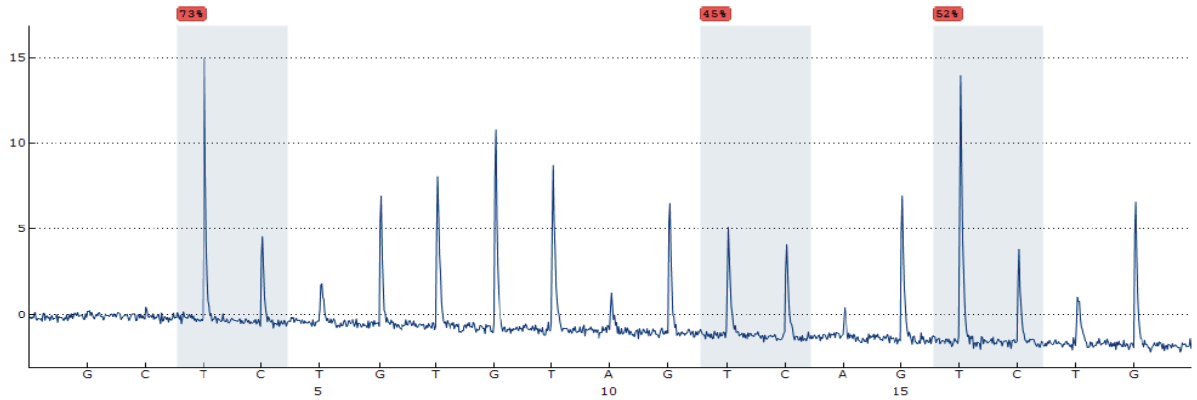
1) Transfer homogenized samples into fresh 1.5mL microcentrifuge tubes.
2) Add 200 ul of chloroform, vortex thoroughly to mix and incubate at room temperature for 15 minutes.
3) Centrifuge at 12,000xg at 4^{o}C for 15 minutes.
4) Transfer the clear aqueous phase into fresh tubes. Be careful to not touch the white layer.
5) Add 0.5mL Propanol to aqueous phase – should see a precipitate band.
6) Invert the tubes and incubate for 10 minutes.
7) Centrifuge at 12,000xg at 4^{o}C for 10 minutes.
8) Remove supernatant leaving the pellet.
9) Add 1mL of 75% ethanol.
10) Vortex to disrupt the pellet (gentle to flick of pellet).
11) Centrifuge at 7,500xg at 4^{o}C for 5 minutes.
12) Remove supernatant (Do not disrupt the pellet).
13) Leave to dry for 10-20 minutes (Do not dry completely). Pellet is white when enthaol and clear when dry.
14) Re-suspend in 20-50uL EDTA storage solution.
15) Heat sample for 10 minutes at 55-60^{o}C.

Appendix I – Failed validation of LINE-1 assay

See below example pyrograms for the commercially available LINE-1 pyrosequencing assay. Top pyrogram displays result for unmethylated control sample, bottom pyrogram displays result for fully methylated control sample.

The software marked both samples as failed (methylation estimation in red) based on the peak height value obtained being lower than the cut off value of 10 RLU (typical values for peak heights when adding a single base are ~50 RLU). Even though the assay mark samples as failed, an estimate of methylation is provided. The mean methylation percentage of the three CpG sites of the LINE-1 assay in the unmethylated sample was 48%; whereas, the mean methylation of the fully methylated sample was 56%. The lack of range (only 8%) between the fully methylated and unmethylated samples provides more evidence that the assay cannot be used for the accurate determination of methylation.





Appendix J – Pyrosequencing assay sequences and example pyrograms

Shown below are the key details about each of the self-designed pyrosequencing assays used in this thesis. Details include 1) original (non-bisulfite converted) genomic sequence indicating the location of TSS, first exon and the CpG sites analysed; 2) assay design report detailing the primer sequences, assay design score, sequence to analyse, bisulfite-converted sequence with marked primers and dispensation order; and 3) an example pyrogram for the assay for a fully methylated control sample.

Key for below assays:

Genomic sequence: TSS indicated by turquoise highlighted sequence; First exon indicated by light green highlighted sequence; CpG sites analysed by the assay are highlighted in blue with orange text; Non-analysed CpG sites highlighted in yellow with red text.

Sequences to analyse / sequence with marked primers: **bases in orange** indicates bisulfite converted cytosine resulting in thymine in the PCR amplified sequence. A Y in the nucleotide sequence indicates a variable position (cytosine or thymine).

Dispensation order: **TC nucleotides in blue** indicates a variable position for methylation determination. **Cytosine marked in orange** indicates the inclusion of a control dispensation to identify contamination with non-bisulfite converted DNA. Both of these features are also marked on the example pyrograms.

Assay: *PPARGC1A*

NCBI Reference Sequence: NC_000004.12 CHR 4: 23891077-23888904 GRCh38.p7 Primary Assembly

Sequence 1kb upstream of TSS

AAGCTCAGGAATTGAATATTTCTGCTAATAGTGTGTTGGTATTTTTCCCTCAGTT
CACAGACATTCTTGATTTCAAAACGCAAACTACACAACCCAGGGCACTAGGGTT
GGAATTCAATGTTTATTCAAAAAGGCACCCTAAGGCAGTTAGGGAGGAAACGCT
ACATGTATGAAAAATAGGAGCCGGGAATCAAAGCTGATCTGAGCAGAGCAGCA
GCGACTGTATTTACTAACACTTGTTTTCTGGGAGCCTATGAGAGAAATGGAAAT
AATTAGAAGGAAGCTGAAAGGATGGGGTTTTGTGGCTTGTTCTCCTTATATGGA
GCAAAGAAAACTGCAGCAACTCTTCGGGAGCTGGTATTCCCTACTGCCATGGG
GGCAGCCGAATTCTGGGTGGAGGAGTTTGTTTATACCTTAACACATACAGGCTA
TTTTGTTGATTAAACAAGCAAAAAAAAAAAAAAAAAAAAAAAAAGCCCCGTTTGC
GCTTTCAAACACTCCCTCAATGAGAAAATGTCTCATAAAAATGCATCATGTGATA
AGCTCTTGCTTTAGTCCCAAACTGAGCTTGAGTCCACTTGGAGATCTTAGAATTA
AAGAGTTCTTAGGGAATACACGTTTTAGCTAAGAATATAGTTACTCTGTCATGAA
ACAGGGAGCTTTGCCACTTGCTTGTTTTGGAAGGAAAATAAATTAAAAAAAGATT
GCAGGGGATTTTGGTTATTATATGGCCAGGGCTCCGTTTAGAGTCTGTGGCATT
CAAAGCTGGCTTTAATCACAGCATGATGCTTGAAGCCTCCAAAAGTCTAAGTGT
TTCCTTTCTTTCTTTCTTTTCTTTTTCTTTCTTTTTTTTTTTTTTTAAAGCGTTACTT
CACTGAAGCAGAGGGCTGCCTTTGAGTGACGTCACGAGTTAGAGCAGCAAGCT
GCACAGGGGAAGGGAGGCTGGGTGAGTGACAGCCCAGCCTACTTTTTAATAGC
TTTGTCATGTGACTGGGGACTGTAGTAAGACAGGTGCCTTCAGTTCACTCTCAG
TAAGGGGCTGGTTGCCTGCATGAGTGTGTGCTCTGTGTCACTGTGGATTGGAG
TTGAAAAAGCTTGACTGGCGTCATTCAGGAGCTGGATGGCGTGGGACATGTGC
AACCAGGACTCTGAGTCTGTATGGAGTGACATCGAG

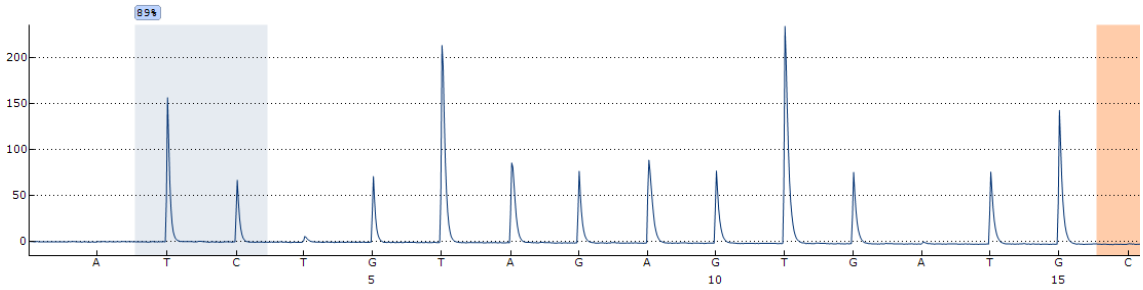
Primer Set 1			Score: 93 Quality: High	
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**
PCR	F1	TGTAGGGGATTTTGGTTATTATATGGT	27	61.1
PCR	R1	ACCAACTTTAAATACCACAAACTCTA	26	59.6
Sequencing	S1	GGTTATTATATGGTTAGGGT	20	46.1
Target Polymorphisms	Position1			
Sequence to Analyze	TTYGTTTAGA GTTTGTGGTA TTTAAAGTT			

Sequence with marked primers

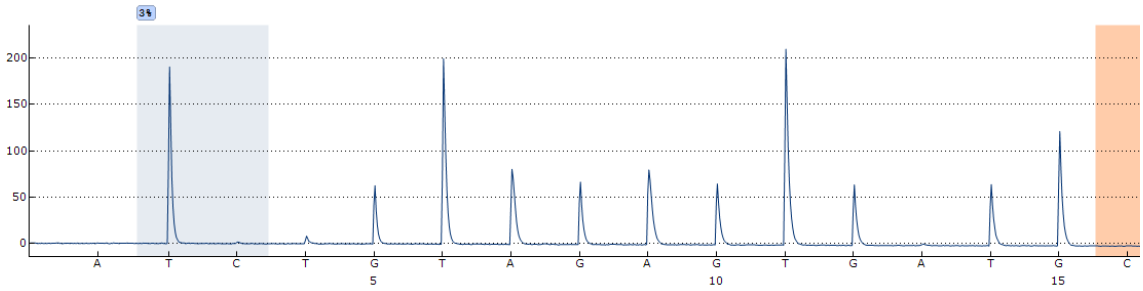
```
    TGTAGGGGAT TTTGGTTATT ATATGGT
    |||||||||| |||||||||| |||||||
              GGTTATT ATATGGTTAG GGT
              ||||||| |||||||||| |||
84 TGTAGGGGAT TTTGGTTATT ATATGGTTAG GGTTTYGTTT AGAGTTTGTG GTATTTAAAG TTGGT 149
                                              | |||||||||| |||||||||| |||||
                                              A TCTCAAACAC CATAAATTTC AACCA
```

Dispensation order: ATCTGTAGAGTGATGCT

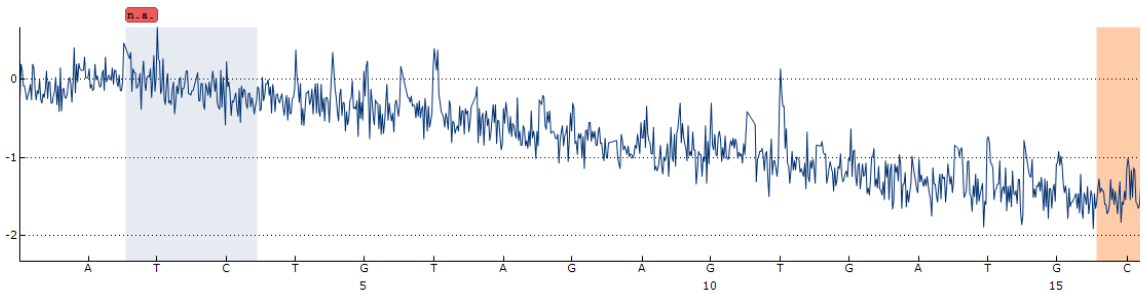
100% methylated bcDNA



0% methylated bcDNA



Non-bcDNA



Assay: *PPARGC1A* ALT

NCBI Reference Sequence: NC_000004.12 CHR 4: 23891077-23888904 GRCh38.p7 Primary Assembly

Sequence 1kb upstream of TSS

AGTCGAAAAGAACTCCGAGTGTTTTTTGAGAAGGGTTGACCTCCTTTCAT
GCCACTTACTGTGAATTAAAGCTGCGAGGGAGAACTAGTTTGGGCATGTGGTA
CAGCACTTAGCGCCACCGCTGCCAGGGAACAAGATTTGTGTTTCTAAGGAGAT
ACAACAAAAAGGAGAATGCTTTAAGAGCCAGCGGCTGTCAGAGTGTAAAAGTAT
CTATGTTCAGAAAGGGAAATAGAAGCTGAATTAAGTAATTTTATACACACGTTAT
GGGGTGCCCTTCTGTGAGTAATTCTTAAATTCAAAAGCAAATAGTTGTGACCAC
AAAGGGCTTAGTACATCTCAGCTGATTTTAGCTCTCAGACTCAAACTGGATATG
ATTGGTATTGACCTGCTCTGTGCATGTGATTTTAAGATGGTCTGACTCTTGTCTG
TCATTCTGTTGGGTTATATTAATAACAGGGGACAAAGGGTGAAATAATCCAGTAA
AGTTTTGAGCAGCCACTTGACAACGTATTCCAAATAAATGAGAGGAGGAAAACC
CTAGCTCTACCAACTGGGGCCATAAAACAGAGTCTTCTACACTCTCTTTAATGTC
AATATTAACCAGTTTGCAAATGTTCAGTCAGTTATGGGCATGGATGGATGTCCT
GAATGGGTTCCCGGGATAAAGTGTCATCATAGGACAGAAATCACAGGGAGAGT
GCACCAAGGAAAAATTACAGTACTGCTATATTTACTTAGTGCCTCTGAACTAGG
GTTTTATTTTCCACGGGTTGGAAAGGGAACCACCTGTCTCAATTGCTGATGTCA
GAGAGCTCCCTCGAGACACAGGGCTGCTGGAAAGCACATGATACTGTACATAT
TTGCTCTTACGTTCGTATCTGGCTAAGATTGGGTTTCAGATTTGTGCCCTATTGT
GGAGTTCATTTAGTAGTGACTCTGAGATGCCCTCCCACGTCACCATGCCCTTGT
GAATTAAAAAGTGGCCTGCCGAAGCCCTTGTTGTGAGTGTTCCCTCATCTCATG
ATACCAGTATTTGCACTGCAGTAAAATGAATGACACACATGTTGGGGTTATCATC
TATGGATTCAATTTTGAAA

Primer Set 1			Score: 89 Quality: High		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%**
PCR	F1	AAGGGAATTATTTGTTTTAATTGTTGATG	29	59.3	24
PCR	R1	AACACAAATCTAAAACCCAATCT	23	58.6	30
Sequencing	S1	GTTGATGTTAGAGAGTTTT	19	44.4	31
Target Polymorphisms	Position9, Position10, Position11				
Sequence to Analyze	TTYGAGATAT AGGGTTGTTG GAAAGTATAT GATATTGTAT ATATTTGTTT TTAYGTTYG ATTTGGTTAA GATTGGGTTT TAGAT				

Sequence with marked primers

```
     AAGGGAATTA TTTGTTTTAA TTGTTGATGT
     |||||||||| |||||||||| ||||||||||
110  AAGGGAATTA TTTGTTTTAA TTGTTGATGT TAGAGAGTTT TTTYGAGATA TAGGGTTGTT GGAAAGTATA TGATATTGTA TATATTTG



200  TTTAYGTTYG TATTTGGTTA AGATTGGGTT TTAGATTTGT GT 242
                |||||||| ||||||||||
                AACCAAT TCTAACCCAA
                         |||||||||| ||||||||||  ||
                         TCTAACCCAA AATCTAAACA CA
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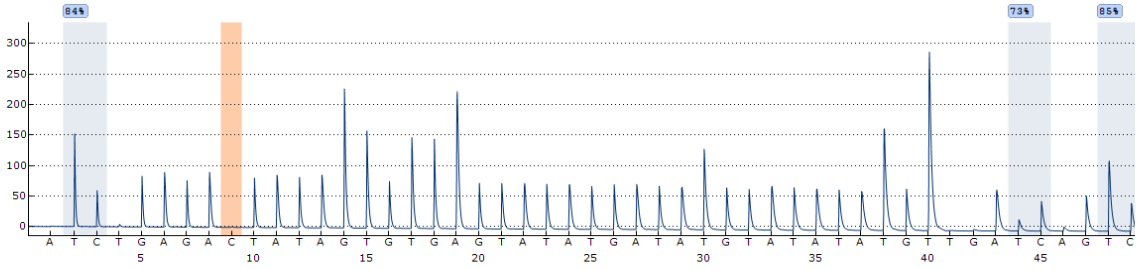
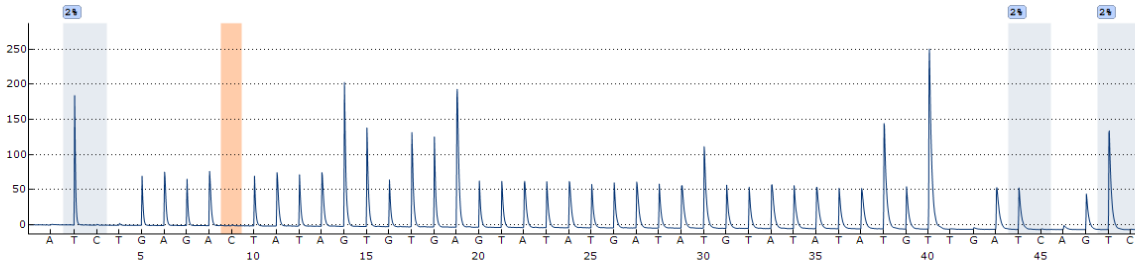
Dispensation order: ATCTGAGACTATAGTGTGAGTATATGATATGATATATTGTTGATCAGTCGTA

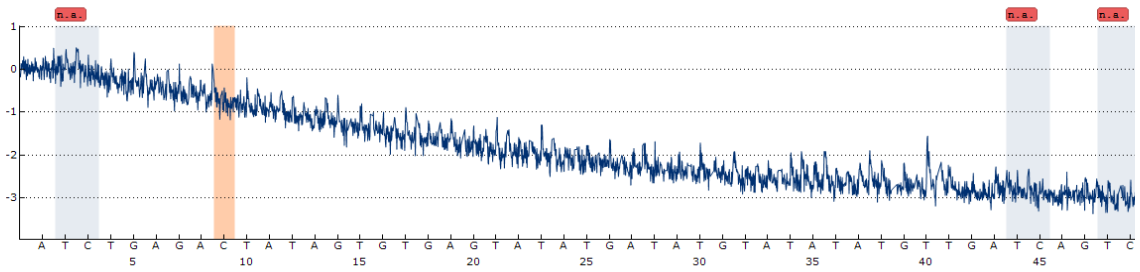
100% methylated bcDNA



0% methylated bcDNA



Non-bcDNA



Assay: *IL6*

NCBI Reference Sequence: NC_000004.12 CHR 7: 22725700-27227281 GRCh38.p7 Primary Assembly

Sequence 1.5kb upstream of TSS

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TAAATGCCCAACAGAGGTCACTGTTTTATCGATCTTGAAGAGATCTCTTCTTAGC
AAAGCAAAGAAACCGATTGTGAAGGTAACACCATGTTTGGTAAATAAGTGTTTT
GGTGTTGTGCAAGGGTCTGGTTTCAGCCTGAAGCCATCTCAGAGCTGTCTGGG
TCTCTGGAGACTGGAGGGACAACCTAGTCTAGAGCCCATTTGCATGAGACCAA
GGATCCTCCTGCAAGAGACACCATCCTGAGGGAAGAGGGCTTCTGAACCAGCT
TGACCCAATAAGAAATTCTTGGGTGCCGACGCGGAAGCAGATTCAGAGCCTAG
AGCCGTGCCTGCGTCCGTAGTTTCCTTCTAGCTTCTTTTGATTTCAAATCAAGAC
TTACAGGGAGAGGGAGCGATAAACACAAACTCTGCAAGATGCCACAAGGTCCT
CCTTTGACATCCCCAACAAAGAGGTGAGTAGTATTCTCCCCCTTTCTGCCCTGA
ACCAAGTGGGCTTCAGTAATTTCAGGGCTCCAGGAGACCTGGGGCCCATGCAG
GTGCCCCAGTGAAACAGTGGTGAAGAGACTCAGTGGCAATGGGGAGAGCACT
GGCAGCACAAGGCAAACCTCTGGCACAGAGAGCAAAGTCCTCACTGGGAGGAT
TCCCAAGGGGTCACTTGGGAGAGGGCAGGGCAGCAGCCAACCTCCTCTAAGT
GGGCTGAAGCAGGTGAAGAAAGTGGCAGAAGCCACGCGGTGGCAAAAAGGAG
TCACACACTCCACCTGGAGACGCCTTGAAGTAACTGCACGAAATTTGAGGATG
GCCAGGCAGTTCTACAACAGCCGCTCACAGGGAGAGCCAGAACACAGAAGAAC
TCAGATGACTGGTAGTATTACCTTCTTCATAATCCCAGGCTTGGGGGGCTGCGA
TGGAGTCAGAGGAAACTCAGTTCAGAACATCTTTGGTTTTTACAAATACAAATTA
ACTGGAACGCTAAATTCTAGCCTGTTAATCTGGTCACTGAAAAAAAATTTTTTTTT
TTTCAAAAAACATAGCTTTAGCTTATTTTTTTTCTCTTTGTAAAACTTCGTGCATG
ACTTCAGCTTTACTCTTTGTCAAGACATGCCAAAGTGCTGAGTCACTAATAAAAG
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CGACCTAAGCTGCACTTTTCCCCCTAGTTGTGTCTTGCCATGCTAAAGGACGTC
ACATTGCACAATCTTAATAAGGTTTCCAATCAGCCCCACCCGCTCTGGCCCCAC
CCTCACCCTCCAACAAAGATTTATCAAATGTGGGATTTTCCCATGAGTCTCAATA
TTAGAGTCTCAACCCCCAATAAATATAGGACTGGAGATGTCTGAGGCTCATTCT
GCCCTCGAGCCCACCGGAACGAAAGAGAAGCTCTATCTCCCCTCCAGGAGC
CCAGCTATGAACTCCTTCTCCACAA

Primer Set 2			Score: 92 Quality: High	
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**
PCR	F2	GGGAAGAGGGTTTTTGAATTAG	22	58.1
PCR	R1	CTCCCTCTCCCTATAAATCTTAATTTAA	28	58.3
Sequencing	S1	TTGAATTAGTTTGATTTAATAAGAA	25	42.4
Target Polymorphisms	Position1, Position2, Position3, Position4, Position5, Position6			
Sequence to Analyze	ATTTTTGGGT GTYGAYGYGG AAGTAGATTT AGAGTTTAGA GTYGTGTTTG YGTTYGT TTTTTTTTAG TTTTTTTTGA TTT			

Sequence with marked primers

```
     GGGAAGAGGG TTTTTGAATT AG
     |||||||||| |||||||||| ||
               TTGAATT AGTTTGATTT AATAAGAA
               ||||||| |||||||||| ||||||||
 57 GGGAAGAGGG TTTTTGAATT AGTTTGATTT AATAAGAAAT TTTTGGGTGT YGAYGYGGAA GTAGATTTAG AGTTTAGAGT YGTGT

147 TTYGTAGTTT TTTTTTAGTT TTTTTTGATT TTAAATTAAG ATTTATAGGG AGAGGGAG 205
                                    |||||||||| |||||||||| ||||||||
                                    AATTTAATTC TAAATATCCC TCTCCCTC
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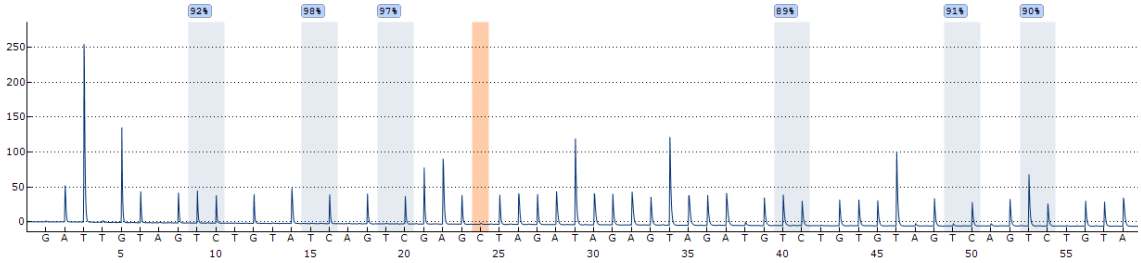
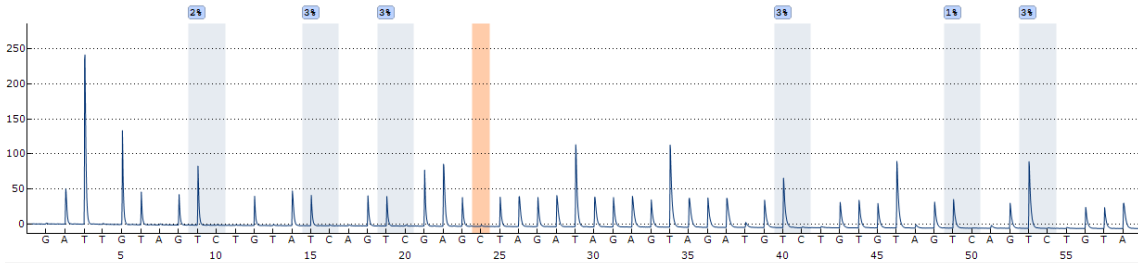
Dispensation order: GATTGTAGTCTGTATCAGTCGAGCTAGATAGAGTAGATGTCTGTGTAGTCAGT

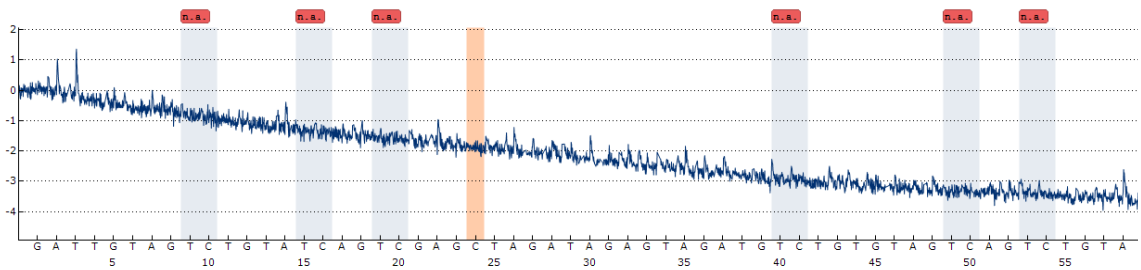
100% methylated bcDNA



0% methylated bcDNA



Non-bcDNA



Assay: *TNF*

NCBI Reference Sequence: NC_000004.12 CHR 6: 31575067 - 31576441

GRCh38.p7 Primary Assembly

Sequence 0.5kb upstream of TSS and downstream on Exon 1

CAGGCCTCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCA
AGGACTCAGCTTTCTGAAGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATC
CTGTCTGGAAGTTAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAGAAATG
GAGGCAATAGGTTTTGAGGGGCATGGGGACGGGGTTCAGCCTCCAGGGTCCT
ACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCGGAATCGGAGCAGGGA
GGATGGGGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAA
ATCCCCGCCCCCGCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCAC
TACCGCTTCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCT
GGTTGAATGATTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACATATAAAGGCA
GTTGTTGGCACACCCAGCCAGCAGACGCTCCCTCAGCAAGGACAGCAGAGGA
CCAGCTAAGAGGGAGAGAAGCAACTACAGACCCCCCCTGAAAACAACCCTCAG
ACGCCACATCCCCTGACAAGCTGCCAGGCAGGTTCTCTTCCTCTCACATACTGA
CCCACGGCTCCACCCTCTCTCCCCTGGAAAGGACACCATGAGCACTGAAAGCA
TGATCCGGGACGTGGAGCTGGCCGAGGAGGCGCTCCCCAAGAAGACAGGGG
GGCCCCAGGGCTCCAGGCGGTGCTTGTTCCTCAGCCTCTTCTCCTTCCTGATC
GTGGCAGGCGCCACCACGCTCTTCTGCCTGCTGCACTTTGGAGTGATCGGCCC
CCAGAGGGAAGAGGTGAGTGCCTGGCCAGCCTTCATCCACTCTCCCACCCAAG
GGGAAATGGAGACGCAAGAGAGGGAGAGAGATGGGATGGGTGAAAGATGTGC
GCTGATAGGGAGGGATGGAGAGAAAAAAACGTGGAGAAAGACGGGGATGCAG
AAAGAGATGTGGCAAGAGATGGGGAAGAGAGAGAGAGAAAGATGGAGAGACA
GGATGTCTGGCACATGGAAGGTGCTCACTAAGTGTGTATGGAGTGAATGAATG
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TGTGAGAAGAGAGATGGGGGAAGAAACAAGTGATATGAATAAAGATGGTGAGA
CAGAAAGAGCGGGAAATATGACAGCTAAGGAGAGAGATGGGGGAGATAAGGA
GAGAAGAAGATAGGGTGTCTGGCACACAGAAGACACTCAGGGAAAGAGCTGTT
GAATGCCTGGAAGGTGAATACACAGATGAATGGAGAGAGAAAACCAGACACCT

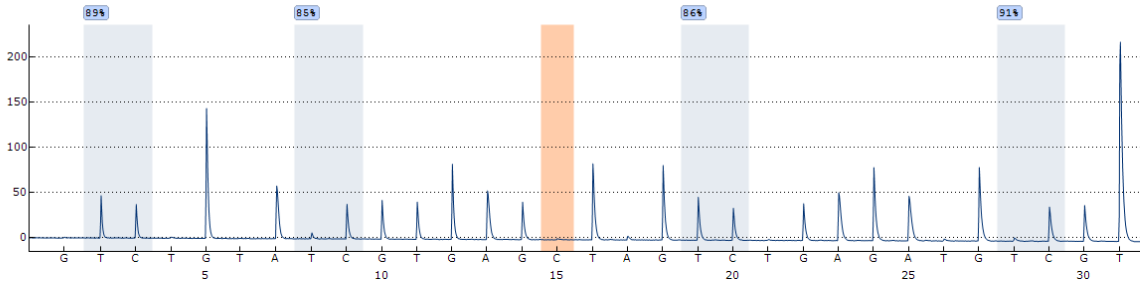
Primer Set 1			Score: 100 Quality: High	
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**
PCR	F1	GGAAAGGATATTATGAGTATTGAAAGTATG	30	58.9
PCR	R1	CTAAAACCCCCCTATCTTCTTAAA	24	58.0
Sequencing	S1	ATTATGAGTATTGAAAGTATGATT	24	44.8
Target Polymorphisms	Position3, Position4, Position5, Position6			
Sequence to Analyze	YGGGAYGTGG AGTTGGTYGA GGAGGYGTTT TTTAAGAAGA TAGGGGGGTT T			

Sequence with marked primers

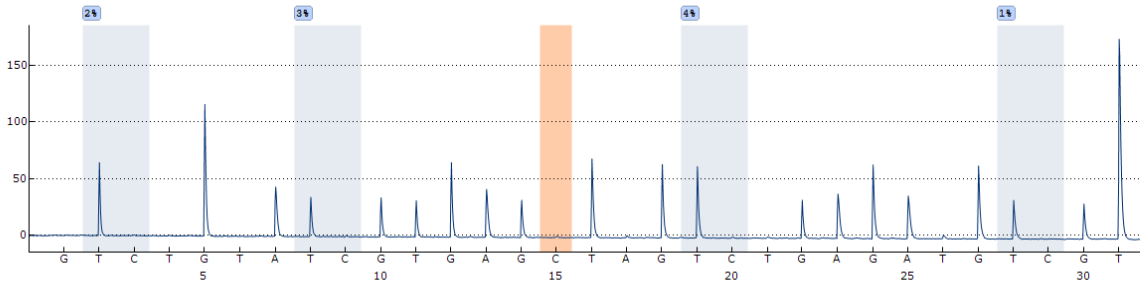
```
     GGAAAGGATA TTATGAGTAT TGAAAGTATG
     |||||||||| |||||||||| ||||||||||
              A TTATGAGTAT TGAAAGTATG ATT
              | |||||||||| |||||||||| |||
111 GGAAAGGATA TTATGAGTAT TGAAAGTATG ATTYGGGAYG TGGAGTTGGT YGAGGAGGYG TTTTTTAAGA AGATAGGGGG GTTTT
                                                                      ||||||| |||||||||| |||||
                                                                      AAATTCT TCTATCCCCC CAAAA
```

Dispensation order: GTCTGTATCGTGAGCTAGTCTGAGATGTCGTTA

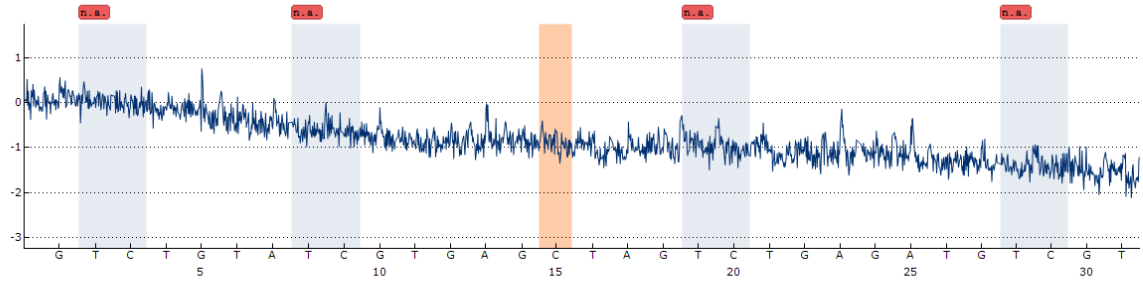
100% methylated bcDNA



0% methylated bcDNA



Non-bcDNA



Appendix K – Sequences for mRNA expression assays

Total PPARGC1A

Primers
For: CAGCCTCTTTGCCCAGATCTT
Rev: TCACTGCACCACTTGAGTCCAC

Sequence
>NM_001330751.1 Homo sapiens PPARG coactivator 1 alpha (*PPARGC1A*), transcript variant 1, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_001330751.1?report=fasta

TCCTCCTGGGAAACCCCTTCCAACCAGGTTTTTTGCGAAAATCAGTGAACTAAT
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CCTGGCTGCTGCTTTGGTAAAAGCTTGCAAGGAGAGAGAGTAACAGCCGCTGG
CGAATCCAGTTTGTGCAAGCAGCATCAGCA{ATGGATGAGACCTCCCCAAGGCT
GGAAGAAGACTGGAAAAAAGTACTTCAGCGAGAAGCAGGCTGGCAG[TGTGCT
GCTCTGGTTGGTGAAGACCAGCCTCTTTGCCCAGATCTTCCTGAACTTGATCTT
TCTGAACTAGATGTGAACGACTTGGATACAGACAGCTTTCTGGGTGGACTCAAG
TGGTGCAGTGACCAATCAGAAATAATATCCAATCAGTACAACAATGAGCCTTCA
AACATATTTGAG]AAGATAGATGAAGAGAATGAGGCAAACTTGCTAGCAGTCCT
CACAGAGACACTAGACAGTCTCCCTGTGGATGAAGACGGATTGCCCTCATTTGA
TGCGCTGACAGATGGAGACGTGACCACTGACAATGAGGCTAGTCCTTCCTCCA
TGCCTGACGGCACCCCTCCACCCCAGGAGGCAGAAGAGCCGTCTCTACTTAAG
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……
TTGCGCAGGTCAAACGAAACTGACTTTGAGCTGTACTTTTGTGGACGCAAGCAA
TTTTTCAAGTCTAACTATGCAGACCTAGATTCAAACTCAGATGACTTTGACCCTG
CTTCCACCAAGAGCAAGTATGACTCTCTGGATTTTGATAGTTTACTGAAAGAAG
CTCAGAGAAGCTTGCGCAGGTAA}CATGTTCCCTAGCTGAGGATGACAGAGGG
ATGGCGAATACCTCATGGGACAGCGCGTCCTTCCCTAAAGACTATTGCAAGTCA
……
ATTCTAAATTTGTACCTATGTGACAGACATTTTCAATAATGTGAACTGCTGATTTG
ATGGAGCTACTTTAAGATTTGTAGGTGAAAGTGTAATACTGTTGGTTGAACTATG
CTGAAGAGGGAAAGTGAGCGATTAGTTGAGCCCTTGCCGGGCCTTTTTTCCAC
CTGCCAATTCTACATGTATTGTTGTGGTTTTATTCATTGTATGAAAATTCCTGTGA
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ATGTTTCAAATCTAAAAAAAAAAAAAAAAA

Key
Amplicon length: 101
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_001330751.1?&feature=CDS

***PPARGC1A* Exon 1a**

Primers
For: ATGGAGTGACATCGAGTGTGCT
Rev: GAGTCCACCCAGAAAGCTGT

Sequence
>NM_013261.4 Homo sapiens PPARG coactivator 1 alpha (*PPARGC1A*), transcript variant 2, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_013261.4?report=fasta

[TAGTAAGACAGGTGCCTTCAGTTCACTCTCAGTAAGGGGCTGGTTGCCTGCAT
GAGTGTGTGCTCTGTGTCACTGTGGATTGGAGTTGAAAAAGCTTGACTGGCGT
CATTCAGGAGCTGG{ATGGCGTGGGACATGTGCAACCAGGACTCTGAGTCTGT
ATGGAGTGACATCGAG][TGTGCTGCTCTGGTTGGTGAAGACCAGCCTCTTTGC
CCAGATCTTCCTGAACTTGATCTTTCTGAACTAGATGTGAACGACTTGGATACA
GACAGCTTTCTGGGTGGACTCAAGTGGTGCAGTGACCAATCAGAAATAATATCC
AATCAGTACAACAATGAGCCTTCAAACATATTTGAG]AAGATAGATGAAGAGAAT
GAGGCAAACTTGCTAGCAGTCCTCACAGAGACACTAGACAGTCTCCCTGTGGA
TGAAGACGGATTGCCCTCATTTGATGCGCTGACAGATGGAGACGTGACCACTG
ACAATGAGGCTAGTCCTTCCTCCATGCCTGACGGCACCCCTCCACCCCAGGAG
GCAGAAGAGCCGTCTCTACTTAAGAAGCTCTTACTGGCACCAGCCAACACTCA
GCTAAGTTATAATGAATGCAGTGGTCTCAGTACCCAGAACCATGCAAATCACAA
TCACAGGATCAGAACAAACCCTGCAATTGTTAAGACTGAGAATTCATGGAGCAA
TAAAGCGAAGAGTATTTGTCAACAGCAAAAGCCACAAAGACGTCCCTGCTCGGA
……
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TTTGAGCTGTACTTTTGTGGACGCAAGCAATTTTTCAAGTCTAACTATGCAGACC
TAGATTCAAACTCAGATGACTTTGACCCTGCTTCCACCAAGAGCAAGTATGACT
CTCTGGATTTTGATAGTTTACTGAAAGAAGCTCAGAGAAGCTTGCGCAGGTAA}
CATGTTCCCTAGCTGAGGATGACAGAGGGATGGCGAATACCTCATGGGACAGC
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ACTCTCTGTACAAAAACAAAACAAAACAACAACAATACAACAAGAACAACAACAA
……
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GCCCTTGCCGGGCCTTTTTTCCACCTGCCAATTCTACATGTATTGTTGTGGTTTT
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Key
Amplicon length: 127
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_013261.4?&feature=CDS

***PPARGC1A* Exon 1b**

Primers
For: CTATGGATTCAATTTTGAAATGTGC
Rev: CTGATTGGTCACTGCACCAC

Sequence
>XM_011513766.1 PREDICTED: Homo sapiens PPARG coactivator 1 alpha (*PPARGC1A*), transcript variant X5, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/XM_011513766.1?report=fasta

[GTGAGTGTTCCCTCATCTCATGATACCAGTATTTGCACTGCAGTAAAATGAATG
ACACAC{ATGTTGGGGTTATCATCTATGGATTCAATTTTGAAA][TGTGCTGCTCT
GGTTGGTGAAGACCAGCCTCTTTGCCCAGATCTTCCTGAACTTGATCTTTCTGA
ACTAGATGTGAACGACTTGGATACAGACAGCTTTCTGGGTGGACTCAAGTGGT
GCAGTGACCAATCAGAAATAATATCCAATCAGTACAACAATGAGCCTTCAAACAT
ATTTGAGGTCCTGGAAGCACAGCCCAGCAGATGACTCACCTCTGGATGGAATC
AACACACAAAGCAGATAAAAAGCTGAACATTAAAGACCACATTGTTAAAGCAGA
GAGCCAAATTGTTCCTCCACCATTCCCTATCCCAGACTTCACATCTAACAAG]AA
GATAGATGAAGAGAATGAGGCAAACTTGCTAGCAGTCCTCACAGAGACACTAG
ACAGTCTCCCTGTGGATGAAGACGGATTGCCCTCATTTGATGCGCTGACAGAT
GGAGACGTGACCACTGACAATGAGGCTAGTCCTTCCTCCATGCCTGACGGCAC
CCCTCCACCCCAGGAGGCAGAAGAGCCGTCTCTACTTAAGAAGCTCTTACTGG
CACCAGCCAACACTCAGCTAAGTTATAATGAATGCAGTGGTCTCAGTACCCAGA
…….
TAACTATGCAGACCTAGATTCAAACTCAGATGACTTTGACCCTGCTTCCACCAA
GAGCAAGTATGACTCTCTGGATTTTGATAGTTTACTGAAAGAAGCTCAGAGAAG
CTTGCGCAGGTAA}CATGTTCCCTAGCTGAGGATGACAGAGGGATGGCGAATA
CCTCATGGGACAGCGCGTCCTTCCCTAAAGACTATTGCAAGTCATACTTAGGAA
TTTCTCCTACTTTACACTCTCTGTACAAAAACAAAACAAAACAACAACAATACAA
CAAATGGCAGCAGTTCCATGAAGACACGCTTAAAACCTAGAACTTCAAAATGTT
CGTATTCTATTCAAAAGGAAATATATATATATATATATATATATATATATATATA
……
GCCAATTCTACATGTATTGTTGTGGTTTTATTCATTGTATGAAAATTCCTGTGATT
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GTTTCAAATCTA

Key
Amplicon length: 153
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: Manually created because the sequence is only predicted on NCBI. Used amino acid sequence of MLGLSSMDSILK to identify the exon 1b and the known second exon as per the other two *PPARGC1A* assays (above).

IL6

Primers
For: GCAGAAAAAGGCAAAGAATC
Rev: CTACATTTGCCGAAGAGC

Sequence
>NM_000600.4 Homo sapiens interleukin 6 (*IL6*), transcript variant 1, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_000600.4?report=fasta

GTCTCAATATTAGAGTCTCAACCCCCAATAAATATAGGACTGGAGATGTCTGAG
GCTCATTCTGCCCTCGAGCCCACCGGGAACGAAAGAGAAGCTCTATCTCCCCT
CCAGGAGCCCAGCT{ATGAACTCCTTCTCCACAAGCGCCTTCGGTCCAGTTGC
CTTCTCCCTGGGGCTGCTCCTGGTGTTGCCTGCTGCCTTCCCTGCCCCAGTAC
CCCCAGGAGAAGATTCCAAAGATGTAGCCGCCCCACACAGACAGCCACTCACC
TCTTCAGAACGAATTGACAAACAAATTCGGTACATCCTCGACGGCATCTCAGCC
CTGAGAAAGGAGACATGTAACAAGAGTAACATGTGTGAAAGCAGCAAAGAGGC
ACTGGCAGAAAACAACCTGAACCTTCCAAAGATGGCTGAAAAAGATGGATGCTT
CCAATCTGGATTCAATGAG[GAGACTTGCCTGGTGAAAATCATCACTGGTCTTTT
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CAGCCTGCTGACGAAGCTGCAGGCACAGAACCAGTGGCTGCAGGACATGACA
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CTTCGGCAAATGTAG}CATGGGCACCTCAGATTGTTGTTGTTAATGGGCATTCC
TTCTTCTGGTCAGAAACCTGTCCACTGGGCACAGAACTTATGTTGTTCTCTATG
GAGAACTAAAAGTATGAGCGTTAGGACACTATTTTAATTATTTTTAATTTATTAAT
ATTTAAATATGTGAAGCTGAGTTAATTTATGTAAGTCATATTTATATTTTTAAGAA
GTACCACTTGAAACATTTTATGTATTAGTTTTGAAATAATAATGGAAAGTGGCTAT
GCAGTTTGAATATCCTTTGTTTCAGAGCCAGATCATTTCTTGGAAAGTGTAGGCT
TACCTCAAATAAATGGCTAACTTATACATATTTTTAAAGAAATATTTATATTGTATT
TATATAATGTATAAATGGTTTTTATACCAATAAATGGCATTTTAAAAAATTCAGCA
A]AAAAAAAA

Key
Amplicon length: 178
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_000600.4?&feature=CDS

TNF

Primers
For: AGGCAGTCAGATCATCTTC
Rev: TTATCTCTCAGCTCCACG

Sequence
>NM_000594.3 Homo sapiens tumor necrosis factor (*TNF*), mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_000594.3?report=fasta

AACTACAGACCCCCCCTGAAAACAACCCTCAGACGCCACATCCCCTGACAAGC
TGCCAGGCAGGTTCTCTTCCTCTCACATACTGACCCACGGCTCCACCCTCTCTC
CCCTGGAAAGGACACC{ATGAGCACTGAAAGCATGATCCGGGACGTGGAGCTG
GCCGAGGAGGCGCTCCCCAAGAAGACAGGGGGGCCCCAGGGCTCCAGGCGG
TGCTTGTTCCTCAGCCTCTTCTCCTTCCTGATCGTGGCAGGCGCCACCACGCTC
TTCTGCCTGCTGCACTTTGGAGTGATCGGCCCCCAGAGGGAAGAG[TTCCCCA
GGGACCTCTCTCTAATCAGCCCTCTGGCCCAGGCAGTCA][GATCATCTTCTCG
AACCCCGAGTGACAAGCCTGTAGCCCATGTTGTAG][CAAACCCTCAAGCTGAG
GGGCAGCTCCAGTGGCTGAACCGCCGGGCCAATGCCCTCCTGGCCAATGGCG
TGGAGCTGAGAGATAACCAGCTGGTGGTGCCATCAGAGGGCCTGTACCTCATC
TACTCCCAGGTCCTCTTCAAGGGCCAAGGCTGCCCCTCCACCCATGTGCTCCT
CACCCACACCATCAGCCGCATCGCCGTCTCCTACCAGACCAAGGTCAACCTCC
TCTCTGCCATCAAGAGCCCCTGCCAGAGGGAGACCCCAGAGGGGGCTGAGGC
CAAGCCCTGGTATGAGCCCATCTATCTGGGAGGGGTCTTCCAGCTGGAGAAGG
GTGACCGACTCAGCGCTGAGATCAATCGGCCCGACTATCTCGACTTTGCCGAG
TCTGGGCAGGTCTACTTTGGGATCATTGCCCTGTGA}GGAGGACGAACATCCAA
CCTTCCCAAACGCCTCCCCTGCCCCAATCCCTTTATTACCCCCTCCTTCAGACA
CCCTCAACCTCTTCTGGCTCAAAAAGAGAATTGGGGGCTTAGGGTCGGAACCC
AAGCTTAGAACTTTAAGCAACAAGACCACCACTTCGAAACCTGGGATTCAGGAA
TGTGTGGCCTGCACAGTGAAGTGCTGGCAACCACTAAGAATTCAAACTGGGGC
CTCCAGAACTCACTGGGGCCTACAGCTTTGATCCCTGACATCTGGAATCTGGA
GACCAGGGAGCCTTTGGTTCTGGCCAGAATGCTGCAGGACTTGAGAAGACCTC
ACCTAGAAATTGACACAAGTGGACCTTAGGCCTTCCTCTCTCCAGATGTTTCCA
GACTTCCTTGAGACACGGAGCCCAGCCCTCCCCATGGAGCCAGCTCCCTCTAT
TTATGTTTGCACTTGTGATTATTTATTATTTATTTATTATTTATTTATTTACAGATG
AATGTATTTATTTGGGAGACCGGGGTATCCTGGGGGACCCAATGTAGGAGCTG
CCTTGGCTCAGACATGTTTTCCGTGAAAACGGAGCTGAACAATAGGCTGTTCCC
ATGTAGCCCCCTGGCCTCTGTGCCTTCTTTTGATTATGTTTTTTAAAATATTTATC
TGATTAAGTTGTCTAAACAATGCTGATTTGGTGACCAACTGTCACTCATTGCTGA
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Key
Amplicon length: 142
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_000594.3?&feature=CDS

DNMT1

Primers
For: TACCTGGACGACCCTGACCTC
Rev: CGTTGGCATCAAAGATGGACA

Sequence
>NM_001130823.2 Homo sapiens DNA methyltransferase 1 (*DNMT1*), transcript variant 1, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_001130823.2?report=fasta

TCCGCGTGGGGGGGGTGTGTGCCCGCCTTGCGCATGCGTGTTCCCTGGGCAT
GGCCGGCTCCGTTCCATCCTTCTGCACAGGGTATCGCCTCTCTCCGTTTGGTA
CATCCCCTCCTCCCCCACGCCCGGACTGGGGTGGTAGACGCCGCCTCCGCTC
ATCGCCCCTCCCCATCGGTTTCCGCGCGAAAAGCCGGGGCGCCTGCGCTGCC
GCCGCCGCGTCTGCTGAAGCCTCCGAG{ATGCCGGCGCGTACCGCCCCAGCC
CGGGTGCCCACACTGGCCGTCCCGGCCATCTCGCTGCCCGACGATGTCCGCA
GGCGGCTCAAAGATTTGGAAAGAGACAGCTTAACAGAAAAGGAATGTGTGAAG
……
AGATGGAGACGAGAAAGATGAGAAGAAGCACAGAAGTCAACCCAAAGATCTAG
CTGCCAAACGGAGGCCCGAAGAAAAAGAACCTGAAAAAGTAAATCCACAGATTT
CTGATGAAAAAGACGAGGATGAAAAGGAGGAGAAGAGACGCAAAACGACCCCC
AAAGAACCAACGGAGAAAAAAATGGCTCGCGCCAAAACAGTCATGAACTCCAA
G[ACCCACCCTCCCAAGTGCATTCAGTGCGGGCAGTACCTGGACGACCCTGAC
CTCAAATATGGGCAGCACCCACCAGACGCG][GTGGATGAGCCACAGATGCTG
ACAAATGAGAAGCTGTCCATCTTTGATGCCAACGAGTCTGGCTTTGAGAGTTAT
GAGGCGCTTCCCCAGCACAAACTGACCTGCTTCAG]TGTGTACTGTAAGCACG
GTCACCTGTGTCCCATCGACACCGGCCTCATCGAGAAGAATATCGAACTCTTCT
TTTCTGGTTCAGCAAAACCAATCTATGATGATGACCCATCTCTTGAAGGTGGTG
TTAATGGCAAAAATCTTGGCCCCATAAATGAATGGTGGATCACTGGCTTTGATG
……
GCCACCGCCCCTGGCCAAAGCCATTGGCTTGGAGATCAAGCTTTGTATGTTGG
CCAAAGCCCGAGAGAGTGCCTCAGCTAAAATAAAGGAGGAGGAAGCTGCTAAG
GACTAG}TTCTGCCCTCCCGTCACCCCTGTTTCTGGCACCAGGAATCCCCAACA
TGCACTGATGTTGTGTTTTTAACATGTCAATCTGTCCGTTCACATGTGTGGTACA
TGGTGTTTGTGGCCTTGGCTGACATGAAGCTGTTGTGTGAGGTTCGCTTATCAA
CTAATGATTTAGTGATCAAATTGTGCAGTACTTTGTGCATTCTGGATTTTAAAAG
TTTTTTATTATGCATTATATCAAATCTACCACTGTATGAGTGGAAATTAAGACTTT
ATGTAGTTTTTATATGTTGTAATATTTCTTCAAATAAATCTCTCCTATAAACCACC
AAAAAAAAAAAA

Key
Amplicon length: 103
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using:
https://www.ncbi.nlm.nih.gov/nuccore/NM_001130823.2?&feature=CDS

DNMT3a

Primers
For: TATTGATGAGCGCACAAGAGAGC
Rev: GGGTGTTCCAGGGTAACATTGAG

Sequence
>NM_175629.2 Homo sapiens DNA methyltransferase 3 alpha (*DNMT3A*), transcript variant 1, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_175629.2?report=fasta

GCAGTGGGCTCTGGCGGAGGTCGGGAGAACTGCAGGGCGAAGGCCGCCGGG
GGCTCCGCGGGCTGCGGGGGGAGGCACTTGACACCGGCCCGGGGAGAGGAG
GGGCCGCTGTCCCTGCGGCCAGTGCTGGATGCGGGGACCCAGCGCAGAAGC
AGCGCCAGGTGGAGCCATCGAAGCCCCCACCCACAGGCTGACAGAGGCACCG
TTCACCAGAGGGCTCAACACCGGGATCTATGTTTAAGTTTTAACTCTCGCCTCC
AAAGACCACGATAATTCCTTCCCCAAAGCCCAGCAGCCCCCCAGCCCCGCGCA
GCCCCAGCCTGCCTCCCGGCGCCCAG{ATGCCCGCCATGCCCTCCAGCGGCC
CCGGGGACACCAGCAGCTCTGCTGCGGAGCGGGAGGAGGACCGAAAGGACG
……
GATGAGAGTGACACTGCCAAGGCCGTGGAGGTGCAGAACAAGCCCATGATTGA
ATGGGCCCTGGGGGGCTTCCAGCCTTCTGGCCCTAAGGGCCTGGAGCCACCA
GAAG[AAGAGAAGAATCCCTACAAAGAAGTGTACACGGACATGTGGGTGGAAC
CTGAGGCAGCTGCCTACGCACCACCTCCACCAGCCAAAAAGCCCCGGAAGAG
CACAGCGGAGAAGCCCAAGGTCAAGGAGATTATTGATGAGCGCACAAGAG][A
GCGGCTGGTGTACGAGGTGCGGCAGAAGTGCCGGAACATTGAGG][ACATCTG
CATCTCCTGTGGGAGCCTCAATGTTACCCTGGAACACCCCCTCTTCGTTGGAG
GAATGTGCCAAAACTGCAAG]AACTGCTTTCTGGAGTGTGCGTACCAGTACGAC
GACGACGGCTACCAGTCCTACTGCACCATCTGCTGTGGGGGCCGTGAGGTGC
TCATGTGCGGAAACAACAACTGCTGCAGGTGCTTTTGCGTGGAGTGTGTGGAC
……
CCTCTTCGCTCCGCTGAAGGAGTATTTTGCGTGTGTGTAA}GGGACATGGGGG
CAAACTGAGGTAGCGACACAAAGTTAAACAAACAAACAAAAAACACAAAACATA
ATAAAACACCAAGAACATGAGGATGGAGAGAAGTATCAGCACCCAGAAGAGAA
AAAGGAATTTAAAACAAAAACCACAGAGGCGGAAATACCGGAGGGCTTTGCCTT
GCGAAAAGGGTTGGACATCATCTCCTGATTTTTCAATGTTATTCTTCAGTCCTAT
TTAAAAACAAAACCAAGCTCCCTTCCCTTCCTCCCCCTTCCCTTTTTTTTCGGTC
AGACCTTTTATTTTCTACTCTTTTCAGAGGGGTTTTCTGTTTGTTTGGGTTTTGTT
TCTTGCTGTGACTGAAACAAGAAGGTTATTGCAGCAAAAATCAGTAACAAAAAAT
AGTAACAATACCTTGCAGAGGAAAGGTGGGAGAGAGGAAAAAAGGAAATTCTAT
AGAAATCTATATATTGGGTTGTTTTTTTTTTTGTTTTTTGTTTTTTTTTTTTGGGTT

Key
Amplicon length: 111
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_175629.2?&feature=CDS

DNMT3b

Primers
For: GGCAAGTTCTCCGAGGTCTCTG
Rev: TGGTACATGGCTTTTCGATAGGA

Sequence
>NM_006892.3 Homo sapiens DNA methyltransferase 3 beta (*DNMT3B*), transcript variant 1, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_006892.3?report=fasta

ACCCACTCCCGCTGCCCCGTCCGGCCCGCGCCGCTTCCTCGCAGCAGCTGCT
CCCGGCTCCGCGGCCGCAGCCCGCGTGGACGCTCCGAGCGCCCCCCGACGG
ACGGGACCGGCTCCCTGGCGGTCGGGCGAGCGGGCGGCAACGCTGCCCGGC
CGGCAGCGCTGGGGTTAAGTGGCCCAAGTAAACCTAGCTCGGCGATCGGCGC
CGGAGATTCGCGAGCCCAGCGCCCTGCACGGCCGCCAGCCGGCCTCCCGCC
AGCCAGCCCCGACCCGCGGCTCCGCCGCCCAGCCGCGCCCCAGCCAGCCCT
GCGGCAGGAAAGC{ATGAAGGGAGACACCAGGCATCTCAATGGAGAGGAGGA
CGCCGGCGGGAGGGAAGACTCGATCCTCGTCAACGGGGCCTGCAGCGACCAG
……
CGCCCGCCTAGCCCAGGACAGCCAGCAGGGGGGCATGGAGTCCCCGCAGGT
GGAGGCAGACAGTGGAGATGGAGACAGTTCAGAGTATCAGGATGGGAAGGAG
TTT[GGAATAGGGGACCTCGTGTGGGGAAAGATCAAGGGCTTCTCCTGGTGGC
CCGCCATGGTGGTGTCTTGGAAGGCCACCTCCAAGCGACAGGCTATGTCTGGC
ATGCGGTGGGTCCAGTGGTTTGGCGATGGCAAGTTCTCCGAG][GTCTCTGCA
GACAAACTGGTGGCACTGGGGCTGTTCAGCCAGCACTTTAATTTGGCCACCTT
CAATAAGCTCGTCTCCTATCGAAAAGCCATGTACCATGCTCTGGAG]AAAGCTA
GGGTGCGAGCTGGCAAGACCTTCCCCAGCAGCCCTGGAGACTCATTGGAGGA
CCAGCTGAAGCCCATGTTGGAGTGGGCCCACGGGGGCTTCAAGCCCACTGGG
ATCGAGGGCCTCAAACCCAACAACACGCAACCAGTGGTTAATAAGTCGAAGGT
GCGTCGTGCAGGCAGTAGGAAATTAGAATCAAGGAAATACGAGAACAAGACTC
GAAGACGCACAGCTGACGACTCAGCCACCTCTGACTACTGCCCCGCACCCAAG
CGCCTCAAGACAAATTGCTATAACAACGGCAAAGACCGAGGGGATGAAGATCA
……
GTGCCCGCCAGAAGCTGCTGGGAAGGTCCTGGAGCGTGCCTGTCATCCGACA
CCTCTTCGCCCCTCTGAAGGACTACTTTGCATGTGAATAG}TTCCAGCCAGGCC
CCAAGCCCACTGGGGTGTGTGGCAGAGCCAGGACCCAGGAGGTGTGATTCCT
GAAGGCATCCCCAGGCCCTGCTCTTCCTCAGCTGTGTGGGTCATACCGTGTAC
CTCAGTTCCCTCTTGCTCAGTGGGGGCAGAGCCACCTGACTCTTGCAGGGGTA

Key
Amplicon length: 113
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_006892.3?&feature=CDS

GAPDH

Primers
For: GCCTCAAGATCATCAGCAATGCCT
Rev: TGTGGTCATGAGTCCTTCCACGAT

Sequence
>NM_001289745.2 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), transcript variant 3, mRNA
https://www.ncbi.nlm.nih.gov/nuccore/NM_001289745.2?report=fasta

GCTCTCTGCTCCTCCTGTTCGACAGTCAGCCGCATCTTCTTTTGCGTCGCCAGG
TGAAGACGGGCGGAGAGAAACCCGGGAGGCTAGGGACGGCCTGAAGGCGGC
AGGGGCGGGCGCAGGCCGGATGTGTTCGCGCCGCTGCGGGCCGAGCCACAT
CGCTCAGACACC{ATGGGGAAGGTGAAGGTCGGAGTCAACGGATTTGGTCGTA
TTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGTGGATATTGTTG
CCATCAATGACCCCTTCATTGACCTCAACTACATGGTTTACATGTTCCAATATGA
TTCCACCCATGGCAAATTCCATGGCACCGTCAAGGCTGAGAACGGGAAGCTTG
TCATCAATGGAAATCCCATCACCATCTTCCAGGAGCGAGATCCCTCCAAAATCA
AGTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAGTCCACTGGCGTCTTCAC
CACCATGGAGAAGGCTGGG[GCTCATTTGCAGGGGGGAGCCAAAAGGGTCATC
ATCTCTGCCCCCTCTGCTGATGCCCCCATGTTCGTCATGGGTGTGAACCATGA
GAAGTATGACAACAGCCTCAAGATCATCAG][CAATGCCTCCTGCACCACCAAC
TGCTTAGCACCCCTGGCCAAGGTCATCCATGACAACTTTGGTATCGTGGAAGG
ACTCATG][ACCACAGTCCATGCCATCACTGCCACCCAGAAGACTGTGGATGGC
CCCTCCGGGAAACTGTGGCGTGATGGCCGCGGGGCTCTCCAGAACATCATCC
CTGCCTCTACTGGCGCTGCCAAGGCTGTGGGCAAGGTCATCCCTGAGCTGAAC
GGGAAGCTCACTGGCATGGCCTTCCGTGTCCCCACTGCCAACGTGTCAGTGGT
GGACCTGACCTGCCGTCTAGAAAAACCTGCCAAATATGATGACATCAAGAAGGT
GGTGAAGCAGGCGTCGGAGGGCCCCCTCAAGGGCATCCTGGGCTACACTGAG
CACCAGGTGGTCTCCTCTGACTTCAACAGCGACACCCACTCCTCCACCTTTGAC
GCTGGGGCTGGCATTGCCCTCAACGACCACTTTGTCAAGCTCATTTCCTG]GTA
TGACAACGAATTTGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCACATGG
CCTCCAAGGAGTAA}GACCCCTGGACCACCAGCCCCAGCAAGAGCACAAGAGG
AAGAGAGAGACCCTCACTGCTGGGGAGTCCCTGCCACACTCAGTCCCCCACCA
CACTGAATCTCCCCTCCTCACAGTTGCCATGTAGACCCCTTGAAGAGGGGAGG
GGCCTAGGGAGCCGCACCTTGTCATGTACCATCAATAAAGTACCCTGTGCTCAA
CCAGTTAAAAAAAAAAAAAAAAAAAA

Key
Amplicon length: 104
Exons: Sequence between [ ]
CDS: Sequence between { }
Identified using: https://www.ncbi.nlm.nih.gov/nuccore/NM_001289745.2?&feature=CDS#feature_NM-001289745.2_exon_0

Appendix L – Standard curves for individual CpG sites

Shown below are the standard curves produced between the expected input methylation and the observed methylation percentage for the validation of the individual CpG sites for the *PPARGC1A* ALT, *IL6* and *TNF* assays used in the present thesis. Specific site analysed can be identified by the title of the individual figure (Name of assay followed by CpG site distance from the TSS).

Figure 1:*PPARGC1A* ALT CpG sites:

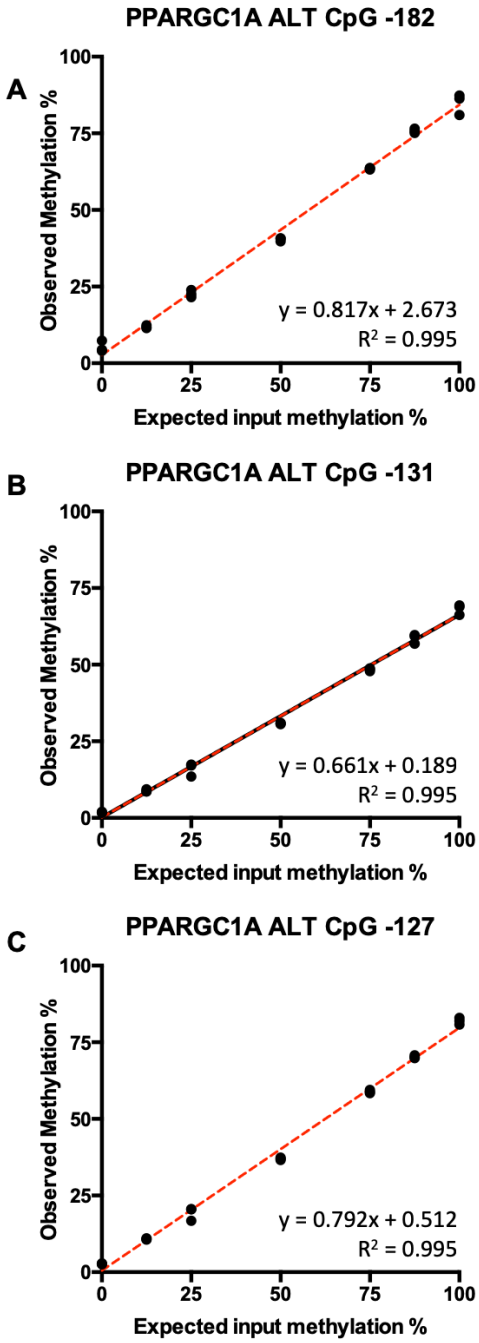


Figure 2: *IL6* CpG sites

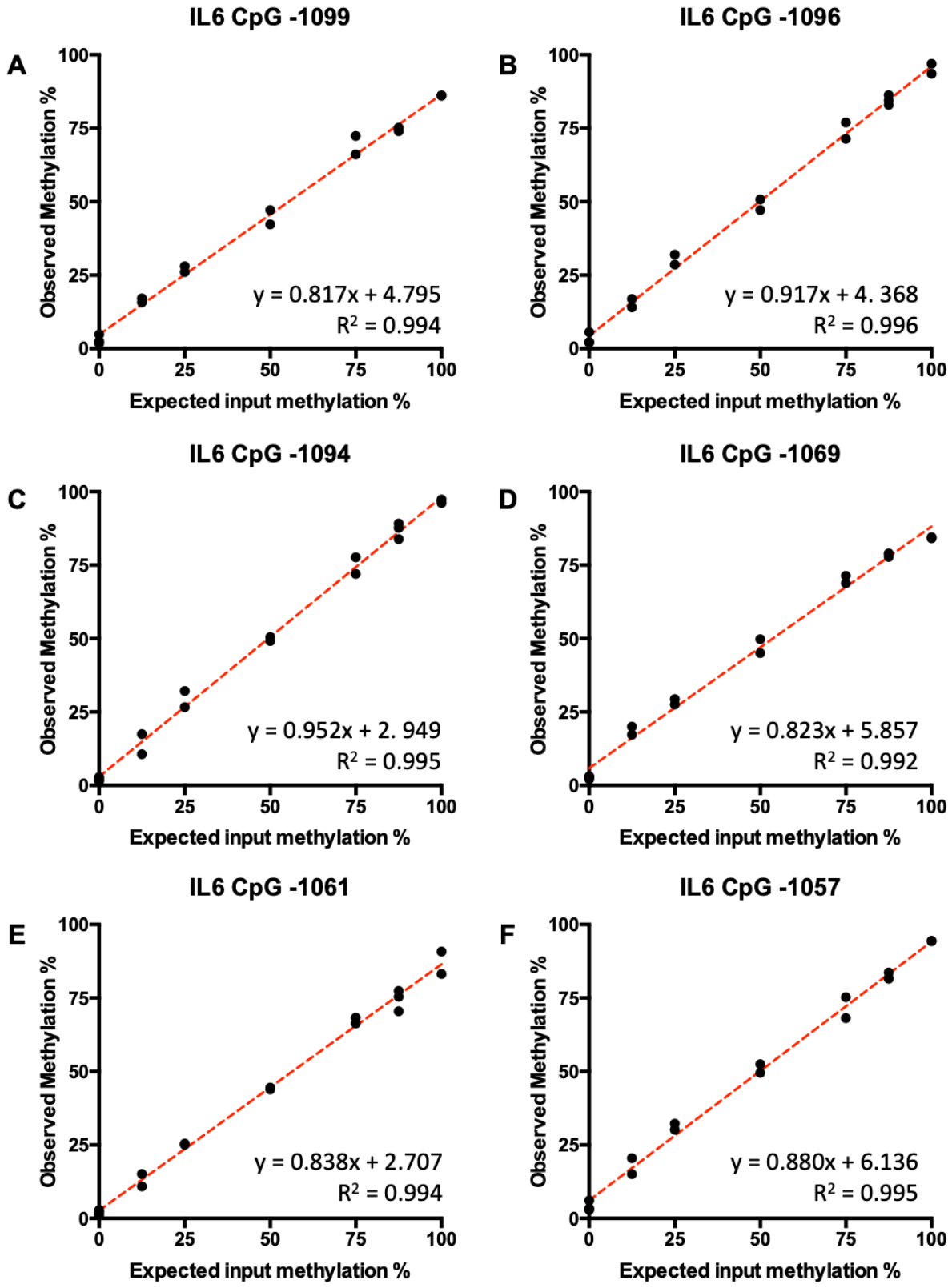
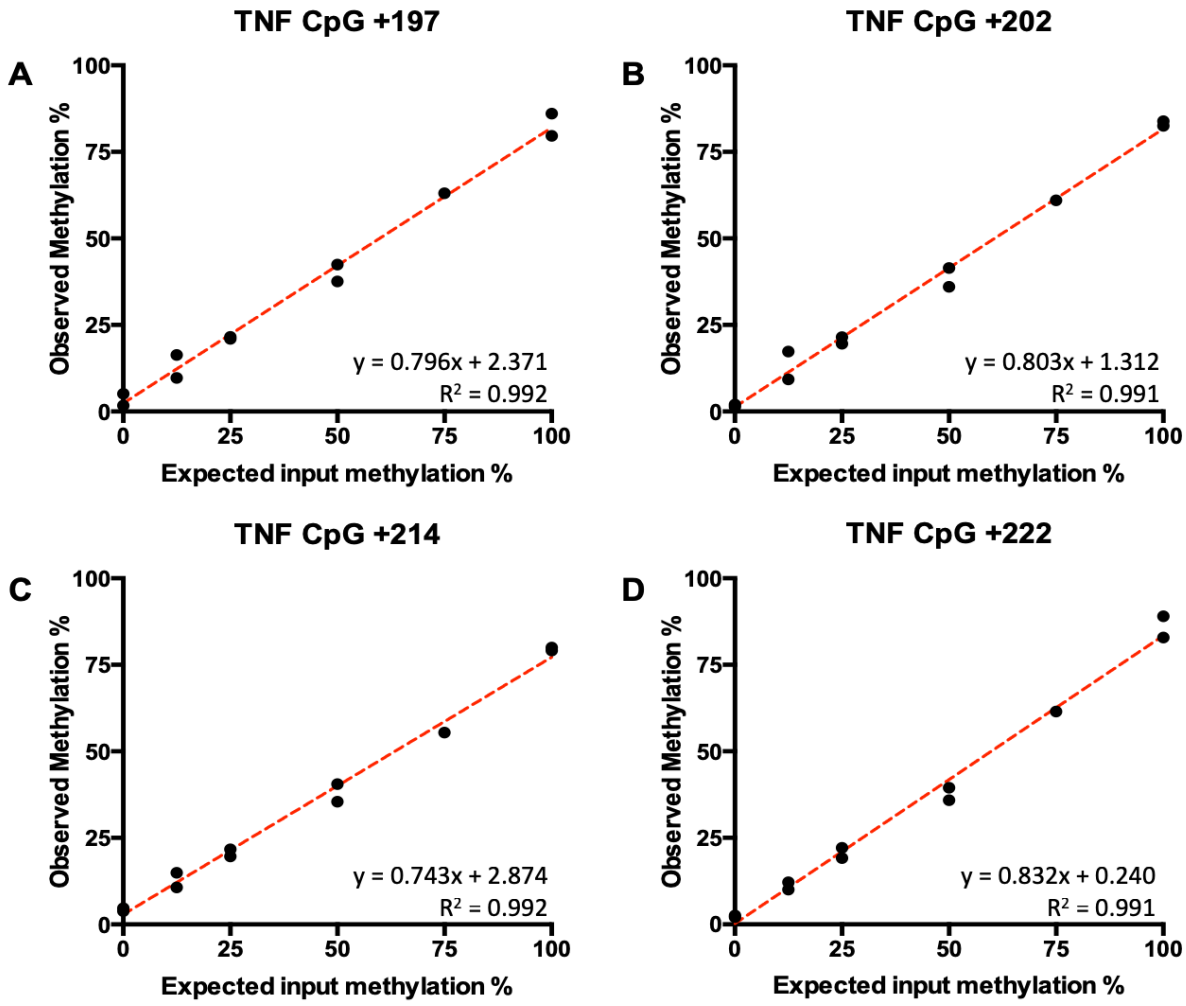


Figure 3: *TNF* CpG sites



Appendix M – qPCR melt curves for mRNA expression

Example melt curves produced for each of the mRNA expression assays used in this thesis. The generation of a single peak indicates that each of the assays specifically amplifies a single transcript.