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AN INVESTIGATION INTO EXERCISE-INDUCED MODIFICATIONS TO DNA METHYLATION-REGULATORY ENZYMES IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Steven Horsburgh

PhD Thesis

2016

AN INVESTIGATION INTO EXERCISE-INDUCED MODIFICATIONS TO DNA METHYLATION-REGULATORY ENZYMES IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Steven Horsburgh

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Abstract

DNA methylation, an epigenetic modification which can regulate gene transcription independently from alterations to the nucleotide sequence, can be manipulated by lifestyle factors such as diet and exercise, hypothetically reversing aberrant DNA methylation associated with disease pathogenesis. The underlying mechanisms by which these changes occur are currently poorly characterised, however, *in vitro* data suggest that inflammatory mediators are involved. Furthermore, regular exercise appears to reduce inactivity-associated systemic inflammation, possibly by alterations to the methylome, thereby suggesting a cyclic relationship between exercise, inflammation, and epigenetic modification.

The aims of this research programme, therefore, were to: characterise the acute changes that occur to the *de novo* DNA methyltransferases following exercise in peripheral blood mononuclear cells (PBMCs), and the role of exercise-induced systemic inflammation in this process; investigate how these changes then translate into functional modifications to the methylome; and to determine whether a training programme utilising sedentary individuals manipulates DNA methylation of genes involved in chronic systemic inflammation associated with physical inactivity.

Pilot investigations corroborated previous *in vitro* data that recombinant IL-6 is able to regulate nuclear concentrations of DNMT3A and DNMT3B in PBMCs. In order to isolate the influence of circulating proteins independently from genetic polymorphisms that may influence susceptibility to epigenetic change, cells were stimulated with exercise-conditioned plasma following intense endurance exercise which elicited significant alterations in nuclear concentrations of DNMT3A and DNMT3B. Eccentric exercise, which is typically not associated with elevations in circulating cytokines, did not cause any significant changes in nuclear or cytoplasmic DNMT concentration, or global DNA methylation; this supports the hypothesis that transient systemic elevations in inflammatory cytokines are important regulators of epigenetic modifications associated with exercise. Lack of transcriptional changes in *DNMT3A* following both exercise training and an acute maximal bout suggests that, in line with *in vitro* data, that the observed elevations in nuclear DNMT concentration are largely due to cellular relocalisation and not gene expression of this enzyme. It remains to be elucidated whether the training regime, and the subsequent response to an acute maximal bout, is able to elicit differential methylation of *IL6, NFκB2,* and *ASC*, however, *in vitro* stimulation of PBMCs with the cytokines IL-6 and IL-1β did cause significant changes to *IL6* promoter methylation, further supporting the role of these proteins in epigenetic regulation.

The data presented in this thesis support the postulation that exercise-induced changes to DNA methylation in PBMCs likely occur due to systemic elevations of inflammatory proteins, in particular IL-6, which causes manipulation of *de* novo DNMT nuclear concentrations due to cellular translocation of the enzymes themselves. While it was not possible to determine whether exercise directly modified gene-specific methylation, *in vitro* experiments suggest that inflammatory cytokines are able to regulate *IL6* promoter methylation in human PBMCs.

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List of Abbreviations

A – Adenine
AA – Ascorbic Acid
AKT – Protein Kinase B
ANOVA – Analysis of Variance
ASC – Apoptosis-associated Speck-like Protein Containing
BC – Breast Cancer
bp – Base Pair
BP – Blood Pressure
BMI – Body Mass Index
bsDNA – Bisulfite-converted DNA
C - Cytosine
Ca^{2+} – Calcium
CAMK – Ca^{2+}/calmodium-dependent Protein Kinase
cDNA – Complementary DNA
CK – Creatine Kinase
CoG – Control Group
CpG – Cytosine-phosphate-Guanine
CRC – Colorectal Cancer
CRP – C-reactive Protein
DNA – Deoxyribonucleic Acid
DNAme – DNA Methylation
DNMT – DNA Methyltransferase
DOMS – Delayed Onset Muscle Soreness
DTT – Diothiothreitol
ELISA – Enzyme-linked Immunosorbent Assay
eNOS – Endothelial Nitric Oxide Synthase
ExG – Exercise Group
FCS – Fetal Calf Serum
FH – Family History
G – Guanine
GBM – Gene Body Methylation
GM – Global Methylation
GPx – Glutathione Peroxidase
GSH – Glutathione
H_2O_2 – Hydrogen Peroxide
HAT – Histone Acetyltransferase
Hb – Haemoglobin
Hct – Haematocrit
HDAC – Histone Deacetylase
HMT – Histone Methyltransferase
HR – Heart Rate
IBD – Inflammatory Bowel Disease
IFN – Interferon
IL – Interleukin
iNOS – Inducible Nitric Oxide Synthase
K – Lysine
LINE-1 – Long Interspersed Nuclear Element 1
LUMA – Luminometric Methylation Assay
MAPK – Mitogen-Activated Protein Kinase
MAT – Methionine Adenosyltransferase
MBD – Methyl CpG-binding Domain
MBP – Methyl Binding Protein
mdFFQ – Methyl Donor Food Frequency Questionnaire
MeDIP – Methylated DNA Immunoprecipitation
miRNA – Micro RNA
msHRM – Methylation-specific High Resolution Melting
MVC – Maximal Voluntary Contraction
ncRNA – Non-coding RNA
NF-κB - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK – Natural Killer Cell
NSAID – Non-steroidal Anti-inflammatory Drug
PA – Physical Activity
PAEE – Physical Activity Energy Expenditure
PAR-Q – Physical Activity Readiness Questionnaire
PBMC – Peripheral Blood Mononuclear Cell
PIC – Protease Inhibitor Cocktail
PGE_2 – Prostaglandin E_2
PM – Promoter Methylation
PPO – Peak Power Output
qPCR – Quantitative Real Time Polymerase Chain Reaction
RA – Rheumatoid Arthritis
rIL-6 – Recombinant IL-6
RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species
RPE – Rating of Perceived Exertion
SAM – S-adenosylmethionine
SD – Standard Deviation
SF – Synovial Fibroblast
SLE – Systemic Lupus Erythematosus
SSc – Systemic Sclerosis
T1/T2DM – Type 1/Type 2 Diabetes Mellitus
T - Thymine
TAB-seq – TET-assisted Bisulfite Sequencing

TET – Ten-Eleven Translocation Methylcytosine Dioxygenase

TSS – Transcription Start Site

TNF – Tumour Necrosis Factor

U – Uracil

UC – Ulcerative Colitis

$\dot{V}O_{2max/peak}$ – maximal/peak oxygen uptake

$v\dot{V}O_{2max}$ – velocity at $\dot{V}O_{2max}$

Xist – X-inactive specific transcript

5-caC – 5-carboxylcytosine

5-fC – 5-formylcytosine

5-hmC – 5-hydroxymethylcytosine

5-mC – 5-methylcytosine

Publications and Conference Proceedings

Peer-reviewed Journal Manuscripts

Horsburgh, S., Robson-Ansley, P., Adams, R., & Smith, C. (2015). Exercise and inflammation-related epigenetic modifications: focus on DNA methylation. [Review]. *Exercise Immunology Review, 21*, pp.26-41.

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Conference Presentations

Horsburgh, S., Costa, R., Todryk, S., Robson-Ansley, P. (2014). The acute effect of ultra-endurance exercise on DNA methyltransferase concentration. Northumbria University Research Conference, Newcastle upon Tyne, May 2014.

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Horsburgh, S., Todryk, S., Toms, C., Moran, C. N., & Ansley, L. (2015). Effects of exercise-conditioned plasma on nuclear DNMT concentration in human peripheral blood mononuclear cells. *Proceedings of The Physiological Society*, *34*(PC065). Physiology, Cardiff, July 2015.

Horsburgh, S., Todryk, S., Toms, C., Moran, C. N., & Ansley, L. (2015). Effects of exercise-conditioned plasma on nuclear DNMT concentration in human peripheral blood mononuclear cells. North East Postgraduate Conference, Newcastle upon Tyne, October 2015.

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Finally, I must wholeheartedly thank all of the participants that took the time out of their lives; without them, this research simply would not have been possible.

Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas, and contributions from the work of others.

I declare that the word count of this thesis is 43, 460.

Name: Steven Horsburgh

Signature:

Date: 23/10/2016

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1 General Introduction and Literature Review

The term 'epigenetics', translating simply to over or above genetics, is now generally accepted to mean 'the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in the sequence of DNA' (Wu & Morris, 2001). Within sport and exercise sciences, the study of epigenetics is still relatively novel, however, the underlying principles have been discussed and debated for some time. The term epigenetics was first coined by Conrad Waddington in 1942, and appeared to contradict the traditional principles of genetics where early 20th century data supports the Darwinian theory that genotype directly influences phenotype, and no interaction occurs between genes and environment. Jean-Baptiste Lamarck's hypothesis that traits acquired during a lifetime can be passed on to future generations appears to be the first suggestion that characteristics do not simply occur from 'nature' or 'nurture' alone. In light of greater understanding of the genome and transcriptional regulation, Lamarck's theory of heritability is being re-credited somewhat, and many scientific disciplines are now acknowledging and utilising epigenetic techniques within their research.

Given that lifestyle associated morbidity and mortality has reached epidemic proportions, it seems worthwhile to investigate how exercise exerts salubrious effects, and the underlying epigenetic modifications which may regulate this process.

The following review will provide an overview of genetic and epigenetic mechanisms, how aberrations in these processes can contribute to disease pathogenesis, the involvement of inflammation as a possible driver and/or consequence, and the role of exercise in the resolution of chronic inflammation and presumably health beneficial changes in DNA methylation.

1.1 Genetics

Deoxyribonucleic acid (DNA), located within the nucleus of a cell, acts as the template by which transcription and translation of a functional protein occur. The familiar double-helix structure of DNA consists of nucleotides; a deoxyribose molecule bound to a phosphate group on one side, and one of four nitrogenous bases on the opposing side. The double-ringed purine bases Adenine (A) and Guanine (G) pair with the single-ringed pyridimine bases Thymine (T) and Cytosine (C) (A with T, G with C).

Nucleosomes, which consist of ~147 base pairs (bp) of double helix structured DNA wrapped around an octamer of histone proteins, are the packaging units of DNA that form chromatin fibres, and when condensed further, form chromosomes.

The term 'gene' is most commonly used to describe a defined section of DNA that is transcribed, and is composed of promoter and coding regions. The coding regions, known as exons, are not continuous, but rather, are broken up by sections of DNA called introns that are not incorporated

into messenger ribonucleic acid (RNA). Upon binding of a transcription factor to a promoter region, typically located upstream of the gene, RNA polymerases begin to separate the two strands of DNA by cleaving the hydrogen bonds. RNA polymerase then adds RNA nucleotides (Uracil (U) replacing T) to the complementary single stranded DNA nucleotides. If the gene is protein coding, mRNA will exit into the cytoplasm *via* the nuclear pore complex, whereby ribosome-mediated translation into a functional protein will occur.

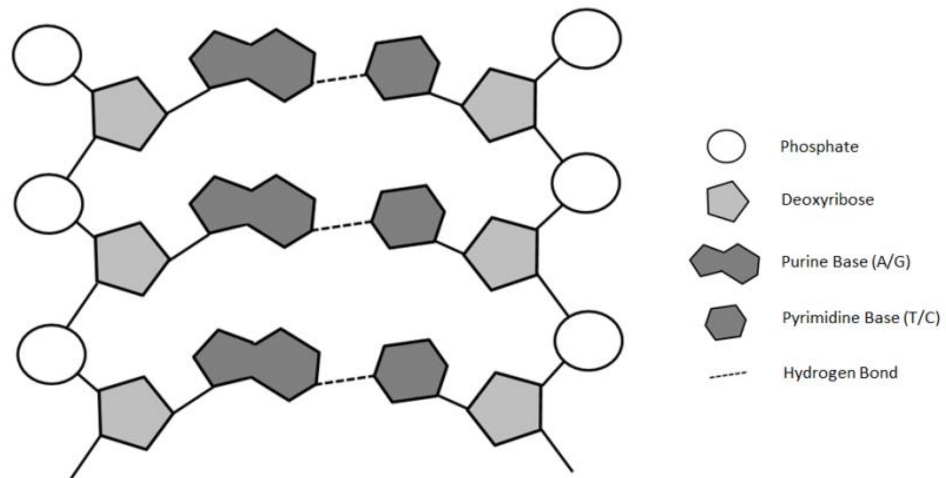

Figure 1.1 *– Schematic of the molecules that make up a short section of DNA.*

Not only did the discovery of epigenetic modifications significantly complicate this process, it also contradicted the long held belief that phenotypes occur as a consequence of genotype or environment, with no interaction between them.

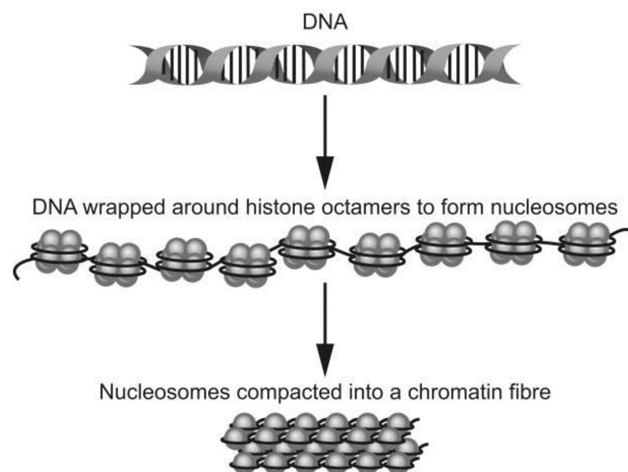

Figure 1.2 *– Structure of a chromatin fibre (image provided courtesy of Abcam Inc.)*

1.2 Epigenetics

Environmental factors such as diet (Choi & Friso, 2010), smoking (Kanherkar, Bhatia-Dey, & Csoka, 2014), and physical activity (PA) (section 1.6), can exert significant influence on an individual's phenotype *via* epigenetic manipulation of gene expression. Research into the underlying molecular mechanisms that regulate these processes is still relatively contemporary, however, there are a number of well-defined epigenetic modifications that form the basis of investigation thus far.

1.2.1 Histone Modifications

Post-translational modifications to the aforementioned histone proteins are key moderators of gene activity, with acetylation and methylation the best characterised, although ubiquitation, phosphorylation, sumoylation, ADP-ribosylation and citrullination also occur.

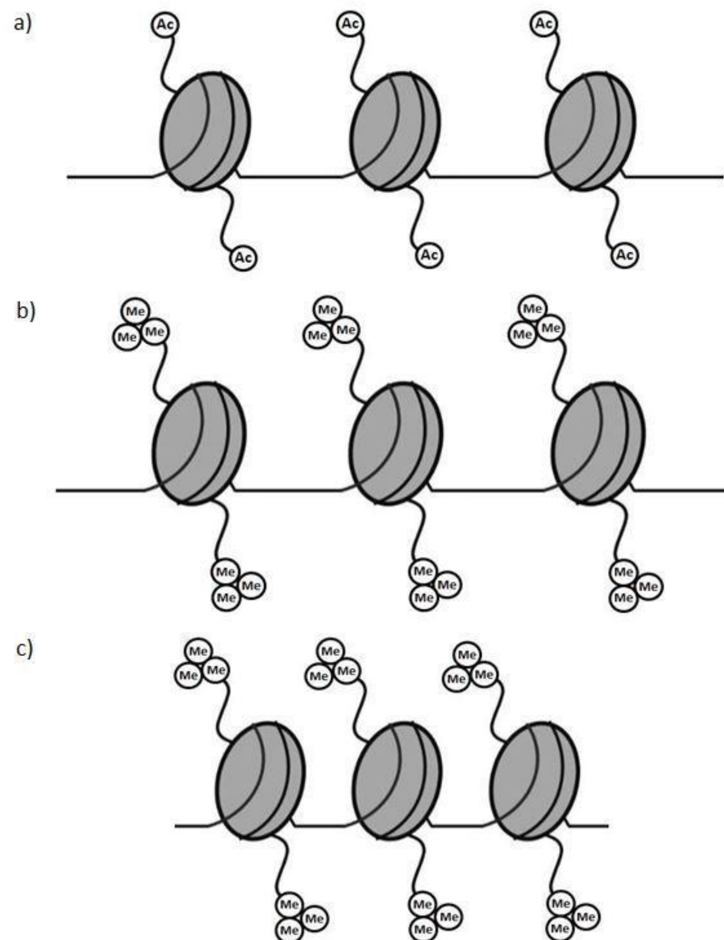

Figure 1.3 *– Histone modifications. a) Histone acetylation; open structure. b) Histone methylation such as H3K4me3; open structure. c) Histone methylation such as Hk9me3; closed structure.*

Acetylation of lysine (K) residues within the N-terminal tail of the histone proteins is associated with gene activation by neutralising the positive charge of lysine, thus decreasing attraction between histones and DNA. Additionally, the attachment of an acetyl group, *via* histone acetyltransferases (HAT), can act as an attachment site for other proteins that are able to recruit chromatin remodelling complexes. Consequently, chromatin is less tightly bound which allows transcription factor binding, thus resulting in gene activation and protein translation. Histone acetylation can be reversed by histone deacetylases (HDAC) however, restoring the positive charge, and thus affinity between lysine and DNA.

In contrast, methylation of histones, catalysed by histone methyltransferase (HMT), can correlate with either transcription or repression depending upon the locus of modification. For example, tri-methylation of lysine residue 4 of histone 3 (H3K4me3) causes gene transcription, whereas tri-methylation of lysine 9 or 27 (H3K9me3/H3K27me3) results in gene silencing. Bannister and Kouzarides (2011) have published a review summarising these modifications and can be referred to for more detail.

1.2.2 Non-coding RNA

Non-coding RNAs (ncRNA) are RNA molecules that are not translated into a protein and can be classified into many sub-groups, including, but not limited to, micro RNAs (miRNA), involved in post-transcriptional gene silencing; piwi-interacting RNAs, which direct DNA methylation at transposable elements; and long non-coding RNAs, which direct epigenetic machinery such as chromatin remodelling complexes. MiRNAs have gained a vast amount of research attention with regard to epigenetics, given the overlap with DNA methylation. For example, alterations in DNA methylation of dysregulated miRNA genes have been reported in a plethora of cancers, including hepatocellular carcinoma cell lines (He et al., 2015), in addition to breast, ovarian, pancreas, colorectal, renal, gastric, lung, cervical, glioblastoma, bladder, endometrial, prostate, melanoma, testicular, and haematological cancers (Lopez-Serra & Esteller, 2012). Furthermore, the role of Interleukin 6 (IL-6) in the modification of DNA methyltransferase 1 (*DNMT1*) expression could be mediated by miRNA-148a and miRNA-152 (Braconi, Huang, and Patel, 2010; Huang, Wang, Guo, & Sun, 2010[a]; Wang et al., 2012[b]). With regard to the *de novo* methyltransferases, miRNA-143 has been shown to target DNMT3A (Ng et al., 2009), miRNA-148 targets the DNMT3B protein coding region (Duursma, Kedde, Schrier, Sage, & Agami, 2008), miRNA-193a represses *DNMT3A* expression (Li et al., 2013), while miRNA-29 appears to be involved in the regulation of both *DNMT3A* and *DNMT3B* (Fabbri et al., 2007; Garzon et al., 2009; Takada, Berezikov, Choi, Yamashita, & Mano, 2009). This demonstrates a complex overlap between different epigenetic alterations and reinforces the fact that each one does not simply occur in isolation.

1.2.3 DNA Methylation

While ‘genome’ refers to an organism’s full set of genes contained within the DNA, the term ‘methylome’ is used to describe the methylation modifications within the genome. DNA methylation, characterised by the DNMT regulated addition of a methyl group to the nucleotide cytosine, creating 5-methylcytosine (5-mC), is the most abundantly studied of the aforementioned epigenetic modifications. This process occurs at CpG dinucleotides (cytosine and guanine separated by phosphate in the linear sequence along DNA), which contribute to less than 1% of the genome (Lander et al., 2001). Clusters of CpG dinucleotides are often located at transcription start sites of genes such as promoter regions, and although DNA methylation has also been found to occur at non-CpG sites (Guo et al., 2014), the process is more commonly reported at the former. Unmethylated sequences with augmented CpG content are known as CpG islands, which were initially defined as regions of at least 200bp, with a GC content of at least 50%, and an observed to expected ratio of 0.6 or greater (Gardiner-Gardner & Frommer, 1987). Similar but slightly redefined cut offs of 500bp, 55%, and 0.65 or greater have been offered more recently by Takai and Jones (2002).

These CpG islands overlap the promoter region of approximately 60 to 70% of human genes (Illingworth & Bird, 2009), although only 7% of genomic CpGs are actually located with CpG islands (Rollins et al., 2006). Furthermore, promoters with lower CpG density are generally reported to be hypermethylated (Weber et al., 2007). It has been suggested that these islands serve to assist transcription factor identification of appropriate promoter sequences (Prestridge & Burks, 1993). Dynamic methylation patterns tend to occur more frequently in CpG shores, regions of low CpG content that flank CpG islands, and CpG shelves, regions that flank shores (Doi et al., 2009; Irizarry et al., 2009) that can also regulate gene expression (Rao et al., 2013).

The effect of methylation at gene promoter or first exon CpG islands is transcriptional silencing of gene expression (Brenet et al., 2011; Rhee et al., 2013), of which the inhibition of transcription factor binding, and the recruitment of methyl-CpG binding proteins (MBPs) which repress the chromatin structure, are key mechanisms (Auclair & Weber, 2012).

Non-promoter i.e. gene-body methylation also occurs with some evidence pointing to a correlation between methylation and expression within these regions (Hellman & Chess, 2007; Laurent et al., 2010; Quierós, Beekman, & Martín-Subero, 2013; Shenker et al., 2015). The mechanisms and role of intragenic methylation remains poorly understood although working hypotheses suggest that alternative promoter usage, alternative splicing, and regulation of ncRNA are all important functional biological consequences (Kulis et al., 2013).

1.2.4 DNA Methyltransferases

A number of DNMTs regulate the methylation process. DNMT1 methylates hemi-methylated DNA, and therefore, has an important role with regards to the maintenance of methylation during cell division. DNMT3A and DNMT3B, on the other hand, show preference toward unmethylated CpG dinucleotides and are both involved in *de novo* methylation during development, albeit at different stages; DNMT3B is the primary enzyme involved in the earlier embryonic stages such as implantation, whereas *DNMT3A* expression is greater in the latter stages of embryonic development (Okano, Bell, Haber, & Li, 1999) as well as during methylation of maturing gametes (Hara et al., 2014; Sakai, Suetake, Shinozaki, Yamashina, & Tajima, 2004). Both enzymes are also expressed in somatic cells, though at very low levels in contrast to embryonic and germ cells.

Another DNMT variant, DNMT3L, despite a lack of methyltransferase activity, assists DNMT3A and DNMT3B by increasing their ability to bind to the methyl donor, S-adenosylmethionine (SAM) (Kareta, Botello, Ennis, Chou, & Chédin, 2006). Although the maintenance of methylation is primarily thought to be regulated by DNMT1, there is evidence to suggest that DNMT3A and DNMT3B also contribute to this process (Chen, Ueda, Dodge, Wang, & Li, 2003). All three of the aforementioned DNMTs are essential in mammalian development as demonstrated by the death of DNMT deficient mice (Lei et al., 1996; Okano et al., 1999). Mutation of the *DNMT3B* gene, and the subsequent loss of methyltransferase activity, can cause ICF (Immunodeficiency, Centromere instability and Facial anomalies) syndrome, an extremely rare recessive disease that affects serum immunoglobulin levels and leads to severe infections, often of the pulmonary or gastrointestinal tracts. Psychomotor and growth retardation are also common symptomologies of ICF patients (Ehrlich, 2003). In addition, DNMT3B has been linked to the fatty acid induced non-CpG methylation of the *PGC1α* promoter observed in Type-2 Diabetes Mellitus (T2DM) patients (Barrès et al., 2009).

1.2.4.1 *DNMT3A* Isoforms and Transcripts

DNMT3A has five protein coding transcripts (“1000 Genomes”, 2015), four of which are recognised by the National Center for Biotechnology Information (NCBI). It is not clear, however, whether all of these transcripts code distinct functional proteins. Chen, Ueda, Xie, and Li (2002) identified an isoform, *DNMT3A2*, initiated from a downstream intronic promoter of the *DNMT3A* gene which lacks the N-terminal 223 amino acid residue. *In vitro*, this isoform has been shown to exhibit similar methyltransferase activity to the main functional protein, however, appears to predominantly be expressed in embryonic, testis, ovary, thymus, and spleen cells, in contrast to the ubiquitously expressed main DNMT3A1 protein strongly suggesting unique functions particularly during development. In HEK293T cells, the two isoforms were reported to share 93% of DNA methylation target sites however (Choi, Heo, Byun, An, Lu, & Yang, 2011). DNMT3A1 does

appear to possess greater DNA binding and methylation activity due to the aforementioned variation in the N-terminal domain (Suetake et al., 2011).

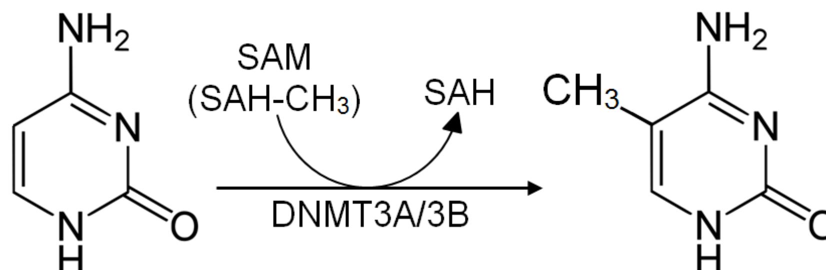

***Figure 1.4** – DNMT-regulated transfer of a methyl group (CH_3) from the methyl donor SAM, converting cytosine (left) into 5-methylcytosine (right), leaving S-adenosylhomocysteine (SAH).*

1.2.4.2 *DNMT3B* Isoforms and Transcripts

DNMT3B has eight protein coding transcripts ("1000 Genomes", 2015), with six of these recognised by the NCBI as coded proteins. DNMT3B1 and DNMT3B2 are considered to be enzymatically active, whereas the other isoforms which occur due to alternative splicing of exons 10, 21 or 22, lack certain motifs and are presumed to be inactive as a consequence. Specifically, *DNMT3B3* lacks part of motif IX (Aoki et al., 2001; Okano et al., 1999), whereas *DNMT3B4* and *DNMT3B5* encode truncated proteins lacking motifs IX and X (Robertson et al., 1999). Chen et al. (2002) were able to identify another variant in embryonic stem cells named *DNMT3B6* which lacks exons 21 and 22, and similarly to *DNMT3B3*, lacks motif IX. Similarly to *DNMT3A*, particular isoforms appear to show tissue specificity; *DNMT3B1* and *DNMT3B6* were the dominant isoforms expressed in embryonic cells, whereas conversely, *DNMT3B2* and *DNMT3B3* were found to be expressed at a greater level in testis, ovary, spleen, thymus, and liver. Interestingly, inactive isoforms such as DNMT3B3 and DNMT3B4 are able to interact with active isoforms; the effects of binding differed drastically, as DNMT3B3 was reported to enhance *de novo* DNA methylation three to four-fold, in contrast to the three-fold reduction in methylation activity caused by DNMT3B4 interaction (Gordon, Hartono, & Chédin, 2013). A frequently expressed aberrant transcript, DNMT3B7, may also interfere with DNA methylation *via* disruption of binding to DNA methylation machinery (Ostler et al., 2007). The effect of these interactions, while considerably lower than the 20-fold enhancement of DNA methylation by DNMT3L, highlights a mechanistic role for proteins that do not necessarily cause *de novo* methylation alone. Unfortunately, the precise functional roles of the various DNMT3B isoforms is still poorly understood in both embryonic and somatic cells (Duymich, Charlot, Yang, Jones, & Liang, 2016).

1.2.5 S-adenosylmethionine Cycle

SAM is involved in a complex pathway (figure 1.5) in order to be resynthesized following donation of its methyl group, as described in figure 1.4. Furthermore, it has been reported that oxidative stress and global DNA hypomethylation are linked due to a number of potential mechanisms; methionine adenosyltransferase (MAT), which catalyzes the addition of adenosine to methionine to yield SAM, exhibits decreased activity in an oxidised environment (Pajares, Durán, Corrales, Pliego, & Mato, 1992). In addition, cystathione β-synthase directs homocysteine towards glutathione (GSH) generation in order for glutathione peroxidase (GPx) to be formed due to its protective role against oxidative damage (Mosharov, Cranford, & Banerjee, 2000). Consequently, homocysteine is less abundantly available for methionine synthesis, and by proxy, SAM synthesis, thereby reducing DNA-methylating capacity (Hitchler & Domann, 2007). Niedzwiecki et al. (2013) showed that a more oxidised blood GSH status was associated with lower global DNA methylation in Bangladeshi adults, which is consistent with this hypothesis. More directly, GSH-depleting agents have been reported to decrease SAM, presumably due to a rebound mechanism whereby methionine is utilised in the transsulfuration pathway in order to replenish GSH, albeit in Syrian hamsters (Lertratanangkoon, Wu, Savaraj, & Thomas, 1997). Similarly, bladder cancer cells treated with hydrogen peroxide (H_2O_2) exhibited hypomethylation in the repetitive element LINE-1 (long interspersed nuclear element), a marker shown to correlate well with other measures of global methylation (Wu et al., 2011), and *RUNX3* hypermethylation, furthering demonstrating that oxidative stress can exert a direct effect on DNA methylation at a global and gene-specific level (Wongpaiboonwattana, Tosukhowong, Dissayabutra, Mutirangura, & Boonla, 2013).

1.2.6 Methyl-CpG Binding Proteins

MBPs play an important role in transcriptional repression and heterochromatin (i.e. closed) structure formation. Three structural families have been identified; methyl CpG-binding domain (MBD), Zinc Finger, and SET and RING finger-associated domain.

MBD1, 2 and 4, which are able to bind to methylated CpG sites, are largely considered to mediate the suppressive effect of DNA methylation. Conversely, MBD3, 5 and 6 do not bind with methylated DNA. MeCP2, another MBD, is thought to interact with a Sin3 and HDAC complex at methylated regions, which results in the repression of the chromatin structure via the mechanism discussed in 1.2.1 (Wade, 2001). There are, however, other mechanisms that can result in chromatin modifications, including interactions between MeCP2 and HMTs (Fuks, Hurd, Wolf, Nan, Bird, & Kouzarides, 2003), such as EZH2 (Mann et al., 2010).

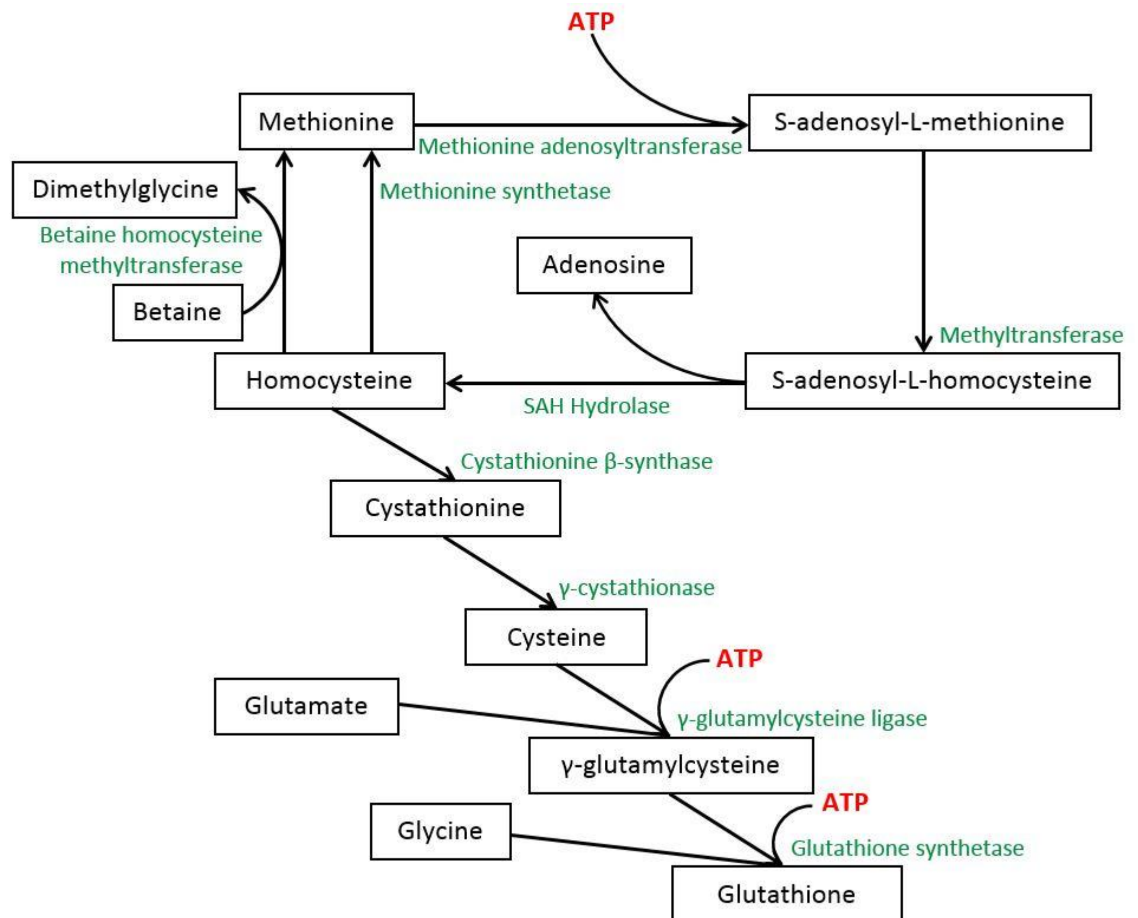

Figure 1.5 *– Schematic representation of how SAM is regenerated following donation of its methyl group during DNA methylation.*

Kaiso, a Zinc Finger protein, is able to differentiate between methylated and unmethylated regions, and acts as a transcriptional repressor. Other Zinc Finger protein family members include ZBTB4, ZBTB38 and ZFP57 (Clouaire & Stancheva, 2008).

UHRF1 and UHRF2 (Ubiquitin-like, containing PHD and RING finger domains) have the ability to bind with methylated DNA through their SRA domains, with the former recruiting DNMT1, and therefore aids the maintenance of methylation. Thus far, UHRF1 is the only MBP that has been shown to bind 5-hydroxymethylcytosine (5-hmC), discussed in section 1.2.7, as well as 5-mC (Buck-Koehntop & Defossez, 2013).

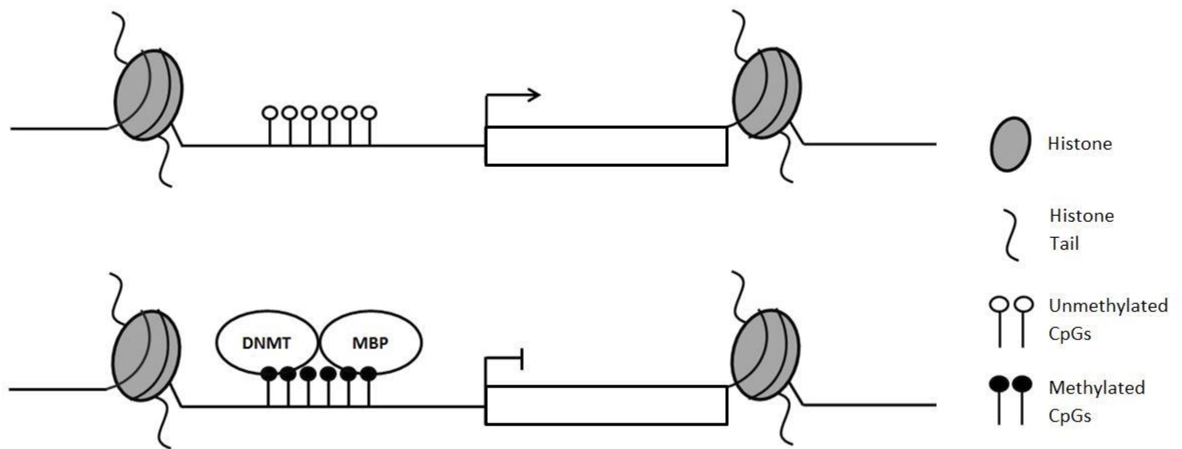

Figure 1.6 *– a) Unmethylated CpG dinucleotides at a gene promoter region - gene is active; b) DNMT-mediated methylation of CpG dinucleotides, followed by MBP recruitment which causes chromatin remodelling and blocking of transcription factors.*

The importance of MBPs in normal developmental regulation is highlighted by Rett's syndrome, a neurodevelopmental disorder of the brain that affects 1 in 10,000 to 1 in 15,000 females, and is commonly mistaken for autism during the early stages of onset. Common symptoms include microcephaly, chorea, ataxia, apraxia and seizures (Van den Veyver & Zoghbi, 2001). Rett's syndrome is caused by germline mutations in MeCP2. Given the role of MeCP2 in binding to methylated portions of DNA and subsequent recruitment of the aforementioned transcriptional repressor complex (Sin3 and HDAC), mutations, of which more than 60 have been identified, generally result in reduced affinity for methylated DNA. Consequently, improper gene suppression occurs (Dragich, Houwink-Manville, & Schanen, 2000). The interactions between the various enzymes and proteins discussed thus far highlights that DNA methylation does not occur in isolation, but rather, is part of a complex cascade of events that regulates epigenetic modification.

1.2.7 DNA Demethylation

An abundance of research has allowed extensive characterisation of both structure and functionality of the enzymes that catalyse DNA methylation. Currently however, less is known regarding the enzymes involved in active demethylation; the removal of a methyl group from 5-mC. If hypermethylation of a gene's promoter region causes suppression of activity, reversal of this process should logically result in gene transcription and protein translation.

The identification of Ten-Eleven Translocation methylcytosine dioxygenase (TET) enzymes was of huge importance in our understanding of the underlying mechanisms that govern the demethylation process. As shown in figure 1.7, TET regulates oxidation of 5-mC to 5-hmC (Tahiliani et al., 2009), then to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) which can both be converted back to unmodified cytosine *via* base excision repair or thymine-DNA glycosylase

(Kohli & Zhang, 2013). The TET family contains three enzymes: TET1, TET2, and TET3; *TET2* is the most widely expressed and promotes demethylation in T cells (Ichiyama et al., 2015) and monocytes (Klug, Schmidhofer, Gebhard, Andreeson, & Rehli, 2013) while *TET3* has also been found to be expressed within peripheral blood leukocytes (Lorsbach, Moore, Mathew, Raimondi, Mukatira, &, Downing, 2003).

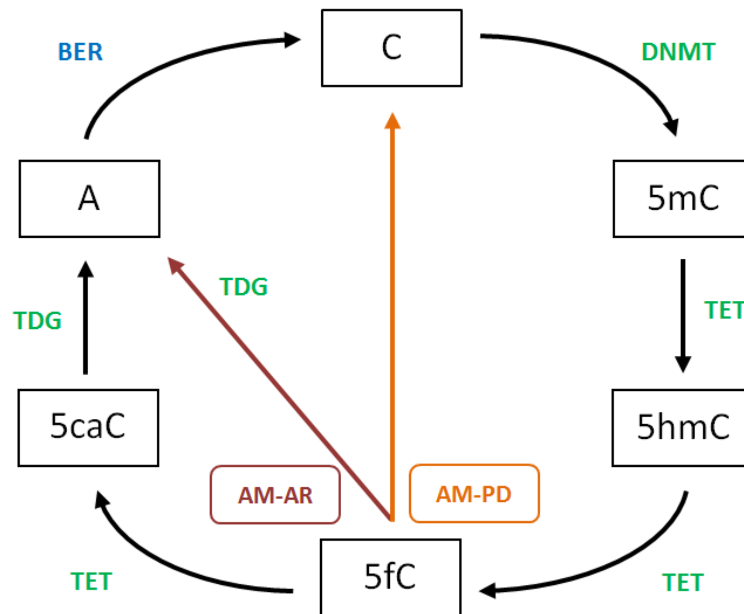

***Figure 1.7** – Demethylation cycle.*

C = cytosine; 5-mC = 5-methylcytosine; 5-hmC = 5-hydroxymethylcytosine; 5-fC = 5-formylcytosine; 5-caC = 5-caboxylcytosine; A = abasic; TDG = DNA glycosylase; AM-AR = active modification-active restoration; AM-PD = active modification-passive dilution; BER = Base excision repair. Enzymes in green.

1.2.8 Role of DNA Methylation

Not only is DNA methylation an important process in normal transcriptional regulation as discussed, it also plays a role in X chromosome inactivation and genomic imprinting (Jin & Robertson, 2011).

X chromosome inactivation, whereby one of the two X chromosomes in the female cell are silenced, is a dosage compensation mechanism in order to ensure that only one set of genes are expressed in each cell. Sharp et al. (2011) have shown that the majority of CpG islands on the inactive X chromosome are hypermethylated, while conversely, genes that do not undergo X chromosome inactivation have lower levels of methylation within the promoter regions. The X Inactivation Centre, a component of the X chromosome, is composed of a number of non-coding RNA genes such as *Xist* (X-inactive specific transcript) (Penny, Kay, Sheardown, Rastan, & Brockdorff, 1996) and *Tsix*, which bind during X chromosome inactivation. An inverse relationship exists between *Xist* and *Tsix* expression (Ahn & Lee, 2008); *Xist* is only expressed in cells with at

least two X chromosomes, and has been found to be essential in the X inactivation process (Penny et al., 1996; Wutz & Jaenisch, 2000), whilst *Tsix* is only expressed in embryonic cells (Migeon, Chowdhury, Dunston, & McIntosh, 2001).

Genomic imprinting, whereby only one working gene is inherited due to epigenetic silencing of a non-expressive gene, is also essential for normal development. If imprinting did not occur, the individual would subsequently have either two active or two inactive copies of a gene, for example in Prader-Willi syndrome, whereby deletion of a group of paternally inherited genes on chromosome 15 results in hypogonadism, infertility, behavioural and cognitive disabilities, hypotonia, hyperphagia, and obesity. Conversely, Angelman syndrome is caused by a maternal deletion on chromosome 15, and is characterised by mental retardation, seizures, ataxia and hyperactivity. Certain cancers such as Wilms' tumour, an embryonic kidney cancer, are also caused by abnormal genomic imprinting. The paternal *IGF2* and maternal *H19* genes are normally expressed due to imprinting, however, this is lost in the cells of Wilm's tumour patients which is thought to cause overexpression of *IGF2* thereby promoting tumourigenesis (Lobo, 2008).

1.3 Aberrant DNA Methylation and Disease

While methylation is clearly an essential component of normal development and transcriptional regulation, aberrant patterns of methylation are associated with a number of diseases. The following section serves as an overview of how epigenetic dysregulation is a contributory factor in disease pathogenesis.

1.3.1 Cancer

Global DNA hypomethylation is common in cervical, prostate, stomach, lung, bladder, oesophageal, colorectal, breast and liver cancers in various cell and tumour types (Lu, Qiu, Hu, Wen, Su, & Richardson, 2006), while promoter CpG hypomethylation and subsequent augmented expression of *EGR1* and *TFF3* occurs during bladder cancer and hepatocellular carcinoma development, respectively (Ogishima et al., 2005; Okada et al., 2005). In addition, hypermethylation of tumour suppressor gene CpG islands, causing suppression and thereby promoting oncogenesis, is well established in a vast number of human cancers (Lu et al., 2006). Dysregulation of DNMTs also appears to be related to various cancers. For example, increased DNMT activity has been reported in colon cancer progression (Issa et al., 1993), while mutations in *DNMT3A* are common in patients suffering from myelodysplastic syndrome (Walter et al., 2011) and acute myeloid leukemia (Ley et al., 2010).

1.3.2 Type 2 Diabetes Mellitus

The link between methylation and metabolic health is also beginning to be acknowledged, particularly within T2DM. Genome-wide analyses have shown that there are a vast number of CpG sites that are differentially methylated in T2D pancreatic islets compared with healthy controls (Dayeh et al., 2014; Volkmar et al., 2012). Similarly, *vastus lateralis* skeletal muscle biopsies taken from men with or without a family history of T2DM identified 65 genes that exhibited differential methylation profiles between the two groups (Nitert et al., 2012). Hypermethylation and decreased expression of *GLP1R*, responsible for glucose-dependent insulin release (Hall, Dayeh, Kirkpatrick, Wollheim, Nitert, & Ling, 2013), *PDX1*, involved in β cell maturation (Yang et al., 2012), and *PGC1α*, a regulator of mitochondrial biogenesis and function (Ling et al., 2008), have been reported in pancreatic islets of type-2 diabetics. Not only has hypermethylation been reported at CpG sites within the insulin gene promoter region, but HbA_{1c} levels were shown to be correlated with methylation, thereby supporting the hypothesis that hyperglycaemia is a regulator of methylation, which had previously been reported in clonal rat β cells (Yang et al., 2011).

1.3.3 Cardiovascular Disease

Global hypomethylation of advanced human atherosclerotic lesions has been reported using High Performance Liquid Chromatography (Hiltunen et al., 2002). Conversely however, elevated levels of methylation measured in the repetitive element ALU, similar to the aforementioned LINE-1 repetitive element method, were positively associated with risk of cardiovascular disease (Kim et al., 2007). This contradictory evidence is likely explained by differences in study population or the measurement techniques.

Inflammatory processes are well recognised in the pathogenesis of atherosclerosis. Briefly, endothelium dysfunction causes adhesion of circulating monocytes, which are then directed toward the intima by chemokines, and thus differentiate into macrophages. Ingestion of oxidized LDL particles by the macrophages forms foam cells which accumulate as coronary plaque. Endothelial Nitric Oxide Synthase (eNOS), the enzyme responsible for nitric oxide production which inhibits leukocyte adhesion to the endothelium, is not only regulated by pro-inflammatory cytokines, but can also influence expression of *NFκB* and *COX2* (Ying & Hofseth, 2007). Interestingly, low levels of methylation have been reported at the *eNOS* promoter in endothelial cells that express the protein, while conversely, high levels of methylation correlate with non-expression (Chan et al., 2004[b]). Additionally, the chromatin structure of the inducible Nitric Oxide Synthase (*iNOS*) promoter can be modified when induced by cytokines (Mellott, Nick, Waters, Billiar, Geller, & Chesrown, 2001), while *iNOS* expressing cells were found to have lower levels of methylation within the promoter region (Chan, Fish, Mawji, Leung, Rachlis, & Marsden, 2005). *iNOS* is expressed in macrophages, endothelial cells and vascular smooth muscle cells, and contributes to

plaque instability due to apoptosis of smooth muscle cells (Boyle, 2005), thus, gene promoter hypomethylation and subsequent expression is unfavourable at this locus.

Estrogen receptors α (*ERα*) and β (*ERβ*), the genes that code for estrogen which has cardioprotective effects (Mendelsohn, 2002), appear to be hypermethylated at the promoter regions in human atherosclerotic plaques (Post et al., 1999) which was corroborated in ascending aorta, carotid and femoral artery lesions (Kim et al., 2007). Similarly, promoter hypomethylation and subsequent elevated protein levels of plasma factor VII, a protein that causes blood coagulation, was found to be associated with atherosclerosis (Friso et al., 2012).

1.3.4 Inflammatory Bowel Disease

The role of DNA methylation in the pathogenesis of the inflammatory bowel diseases ulcerative colitis (UC) and Crohn's disease was identified early on with the finding that methylation of the rectal mucosa was 10 times greater in chronic UC patients compared with healthy controls (Gloria et al., 1996). Cooke et al. (2012) reported similar findings between UC patients and controls. They also found that global hypermethylation had only occurred in inflamed mucosa of UC patients, and not in non-inflamed tissue, highlighting the importance of inflammation as a contributory factor in epigenetic dysregulation. Hypermethylation of the tumour suppressor *DAPK* promoter was shown to result in transcriptional inactivation, a modification related to inflammation associated tumours such as gastric carcinoma (Kang, Zhang, Kim, Bae, & Hyun, 2001). A direct association was also observed between *DAPK* methylation and inflammation in the mucosa of UC patients (Kuester et al., 2010). The direction of causality remains to be elucidated, however, it is possible that chronic inflammation causes hypermethylation of the *DAPK* promoter, which has an overall oncogenic effect *via* the downregulation of *DAPK*. This provides a direct mechanistic link between inflammatory bowel disease (IBD) and the increased risk of colitis-associated cancers.

All of the diseases discussed thus far share a common trait in that they are all associated with inflammatory processes. A logical progression would be then to examine the role of aberrant changes in immune cells given their direct role in immune and inflammatory regulation.

1.4 Disease-associated Genetic and Epigenetic Dysregulation in Leukocytes

Peripheral blood mononuclear cells (PBMCs) are a population composed of any leukocyte located in the blood that has a round nucleus such as T lymphocytes, B lymphocytes, Natural Killer (NK) cells, monocytes, and dendritic cells, with lymphocytes comprising the vast majority. Erythrocytes and polymorphonuclear cells such as neutrophils and eosinophils are removed during fractionation, however, basophils are sometimes present in very small proportions. Peripheral blood cells share more than 80% of the transcriptome with nine major other tissues, including organ-specific genes

(Mohr & Liew, 2007), therefore it is clear that they are a useful, relatively non-invasive, and inexpensive biomarker for investigating genetic and epigenetic perturbations associated with disease.

In addition to the aberrant epigenetic changes that contribute to the pathogenesis of a number of disease states in a vast number of tissues (section 1.3), a large body of evidence demonstrates that epigenetic dysregulation in PBMCs is central in the pathophysiology of various diseases, particularly those associated with inflammation. Not only this, but PBMC gene expression can be used as a predictive or diagnostic marker. For example, PBMC gene expression can be used in the prediction of chronic fatigue syndrome (Kaushik et al., 2005), pancreatic cancer (Baine et al., 2011), and glucocorticoid sensitivity in asthma (Hakonarson et al., 2005), outcome in idiopathic pulmonary fibrosis (Herazo-Maya et al., 2013), and in the identification of post-traumatic stress disorder (Segman, Shefi, Goltser-Dubner, Friedman, Kaminski, & Shalev, 2005). In addition, PBMC gene expression can be used as a diagnostic marker of COPD (Bahr et al., 2013), as 16 genes have been reported to be differentially regulated in COPD compared with controls (Wu, Sun, Chen, Bai, & Wang, 2014). The expression of a number of genes, including *IM2*, *NLRP3*, *ASC*, *CASP1*, *CASP5*, and *IL1β* have been shown to be associated with age (Waldron & Holodniy, 2015), while a vast number of genes are differentially expressed in peripheral arterial disease (Masud, Shameer, Dhar, Ding, & Kullo, 2012). Furthermore, differential expression of a number of genes is able to discriminate between Dengue fever and Dengue hemorrhagic fever (Ubol, Masrinoul, Chaijaruwanich, Kalayanarooj, Charoensirisuthikul, & Kasisith, 2008).

Not only do aberrant alterations to the methylome occur in the variety of tissues discussed above, epigenetic dysregulation in PBMCs is closely linked to a number of diseases, again, with inflammatory components.

1.4.1 Cancer

On a global scale, hypomethylation of PBMC DNA appears to be an early change in carcinogenic processes; in a case-control study, cancer subjects, which included malignancies ranging from hemotalogical, bladder, gastrointestinal, prostate, breast, kidney, lung, and larynx, exhibited lower DNA methylation than controls, measured by Liquid Chromatography Mass Spectometry (Friso et al., 2013). This study highlights the use of aberrant changes to the global methylome as a useful biomarker of cancer risk.

With regard to specific cancers, nasopharyngeal cancer and colorectal cancer (CRC) were both associated with lower LINE-1 methylation (Kitkumthorn, Tuangsintanakul, Rattanatanyong, Tiwawech, & Mutirangura, 2012). On a gene-specific level, methylation at *FOXP3* CpGs were associated with transcriptional suppression of the gene in CRC patients PBMCs and tumour samples (Ganapathi, Beggs, Hodgson, & Kumar, 2014). Due to the role of this gene in the control

of regulatory T cells, suppression as a consequence of epigenetic changes likely explains the previously reported large number of tumour infiltrating FOXP3+ regulatory T cells, which was inversely correlated with outcome, in various cancers (Curiel et al., 2004; Hiraoka, Onozato, Kosuge, & Hirohashi, 2006; Kobayashi et al., 2007). Furthermore, over 27,000 and 15,000 differentially methylated regions were discovered in endometrioid adenocarcinoma and uterine papillary serous carcinoma, respectively (Zhang et al., 2014). The majority of regions possessed promoter or enhancer functions of genes associated with development and disease of the uterus.

Although not strictly a cancer, elevated LINE-1 methylation is associated with cervical intraepithelial neoplasia, an abnormal growth of squamous cells on the cervical lining, which can, however, progress to cervical cancer if untreated (Piyathilake et al., 2011).

1.4.2 Cardiovascular Disease

Lower LINE-1 methylation has been linked to increased risk of ischemic heart disease and stroke in peripheral blood (Baccarelli et al., 2010), while 83% and 75% of subjects that exhibited augmented *ABCG1* and *GALNT2* promoter methylation, respectively, also suffered from coronary heart disease (Peng et al., 2014). A large scale study of over 60,000 Chinese adults reported that global methylation, measured in the repetitive elements ALU and Satellite 2, was augmented with a history of cardiovascular disease in men but not women in peripheral blood leukocytes (Kim, Long, Arakawa, Wang, Yu, & Laird, 2010). Furthermore, global hypomethylation was reported in PBMC samples from *Apoe*$^{-/-}$ mice at six months but not four weeks (Lund et al., 2004). Given the role of apolipoprotein E in the production of lipoproteins, which in turn are involved in atherosclerosis, epigenetic dysregulation of this gene could be a causative event in the early stages of coronary artery disease.

1.4.3 Metabolic Disorders

Similarly to cancer, metabolic disorders are also characterised by epigenetic dysregulation in tissues that directly influence the progression of the disease such as tumours or pancreatic islets, but also in PBMCs.

Type 1 Diabetes Mellitus (T1DM) differs from Type 2 due to the autoimmune destruction of pancreatic β cells, in contrast to lifestyle-induce insulin resistance that characterises Type 2. In an analysis of whole blood, modifications to the methylome have been shown to be associated with proliferative diabetic retinopathy in those with T1DM (Agardh et al., 2015). 349 differentially methylated CpG sites across 233 unique genes were identified, with 79% of these hypomethylated compared with controls. In a prospective cohort, 28 CpG sites were identified in those that then developed retinopathy compared with those that didn't; many of these sites overlapped the genes that were previously identified in the case control analysis. Furthermore, of seven CpG sites in the

INS promoter region of leukocyte DNA, T1DM patients possessed differential methylation in four, although it must be noted that methylation did not correlate with the duration of disease or HbA_{1c} (Fradin et al., 2012).

Discordance in T1DM incidence between monozygotic twins has been reported to be 40 to 50%, and thus, is a useful model to investigate the epigenetic contribution to the disease. An epigenome-wide association study identified 132 differentially methylated CpG sites in CD14+ monocytes isolated from discordant twin pairs which were associated with various genes involved in immune responses and T1DM such as *NFκB1A, TNF,* and *GAD2* (Rakyan et al., 2011). Similarly, in B lymphocytes, 88 CpG sites were found to be differentially methylated between discordant twin pairs, with immune defence and cell signalling pathways being the associated with these differential methylation patterns (Stefan, Zhang, Conception, Yi, & Tomer, 2014). Once this data was then integrated with genome-wide association study data, six genes were shown to overlap; *HLA-E* was the only T1DM gene found to be hypomethylated at a promoter region CpG island, whereas two genes (*HLA-DQA2* and *CD226*) were hypermethylated. The remaining three genes, *HLA-DOB, INS,* and *IL-2RB* were hypermethylated in the majority of twins, but this finding was independent of disease discordance.

In a sample of T2DM patients with diabetic nephropathy, PBMC global methylation was significantly greater in those with albuminuria (Maghbooli, Larijani, Emamgholipour, Amini, Keshtkar, & Pasalar, 2014). Global methylation has also been reported to be elevated in B lymphocytes and natural killer (NK) cells in those with T2DM (Simar et al., 2014).

In a large scale (n > 1,000) case-control analysis, Toperoff et al. (2012) identified a number of differentially methylated regions in leukocytes isolated from T2DM patients. Hypomethylation of a CpG site located within the first intron of the *FTO* gene, associated with T2DM and obesity, was also discovered. Importantly, this finding was significant even when factoring in the sequence polymorphism located in the same region. In addition to this, a prospective study in an independent sample showed that young individuals that later developed T2DM exhibited hypomethylation prior to the onset of symptoms. Similarly, although not using white blood cells but rather whole blood, analysis of a prospective cohort demonstrated that those that did not improve in measures of carbohydrate metabolism exhibited significantly lower LINE-1 methylation (Martin-Nunez et al., 2014). Since reduced insulin sensitivity occurs early on in the progression of metabolic disorders, analysis of the blood or leukocytes could be a predictive biomarker of future diabetes risk.

1.4.4 Autoimmune Disorders

Aberrations in methylation are thought to be a contributory factor in autoimmune diseases such as Systemic Lupus Erythematosus (SLE). Global hypomethylation in T cells has been reported in patients with SLE (Richardson, Scheinbart, Strahler, Gross, Hanash, & Johnson, 1990; Zhu, Liang,

Li, Yang, Xiang, & Xu, 2011) due to decreased ras-MAPK (mitogen-activated protein kinase) pathway signalling and subsequent reduction in DNMT expression (Zhu et al., 2011) and activity (Deng et al., 2001). The gene that Perforin, a cytolytic protein, is transcribed from was reported to be hypomethylated in T cells of patients with active SLE, causing overexpression (Kaplan, Lu, Wu, Attwood, & Richardson, 2004).

Global hypomethylation has been reported in CD4+ T helper cells, (Lei et al., 2009; Qin et al., 2013), concomitantly with augmented expression of *MBD2* and *MeCP2*, and attenuated expression of *DNMT1* and *MBD4* (Lei et al., 2009). Global methylation was also inversely correlated with disease severity, suggesting that epigenetic mechanisms possess a central role in the pathophysiology of the disease. On a gene-specific level, hypomethylation of *IL-10* and *IL-13* has been reported (Zhao et al., 2010[a]). Despite the role of IL-10, and to an extent IL-13, as anti-inflammatory cytokines, aberrant expression of these genes is likely to induce faulty inflammatory regulation.

In addition, active SLE is associated with hypomethylation of regions close to the *ITGAL* promoter, involved in *LFA-1* (lymphocyte function-associated antigen 1) expression (Lu et al., 2002). Since *LFA-1* overexpression is common in active SLE (Yung et al., 1996), this provides a mechanistic link between methylation and the development of autoimmunity.

With a specific focus on PBMCs, Liu et al. (2011) discovered global hypomethylation and augmented *DNMT1* and *MBD2* expression. The differential expression of *DNMT1* between PBMCs and T cells alone highlights the tissue specificity of epigenetic modulation, which is further demonstrated by the patterns of global methylation; the aforementioned global demethylation reported in T cells, CD4+ T helper cells and PBMCs contrasts the global hypermethylation that has been reported in whole blood (Errante, Perazzio, Rodrigues, Ferraz, & Caricati-Neto, 2014).

Systemic sclerosis (SSc) is an autoimmune disease involving fibrosis of the skin which is preceded by inflammation and vasculopathy with an abundance of recent evidence suggesting that aberrant epigenetic mechanisms, particularly in T lymphocytes, play a key role in disease pathogenesis. It appears that miRNAs, long-ncRNAs, histone modifications, and DNA methylation changes are all involved, however, due to the scope of this literature review, only the data concerning DNA methylation will be discussed further. SSc is similar to SLE in that CD4+ global hypomethylation has been reported possibly due to the reduction in the expression of *DNMT1*, *MBD3*, and *MBD4* (Lei et al., 2009). On a gene-specific level, the *CD70* promoter region was hypomethylated concomitantly with an increase in CD70, a member of the tumour necrosis factor (TNF) family that regulates T cell function (Jiang et al., 2012).

Rheumatoid arthritis (RA) is defined as an autoimmune disease characterised by inflammation of synovial joints caused by release of macrophages, lymphocytes and inflammatory cytokines from synovial fibroblasts (SF). Hypomethylation of a number of gene promoters has been reported in

SFs of RA patients, namely, *CXCL12* (Karouzakis et al., 2011), *CHI3L1*, *CASP1*, *STAT3*, *MAP3K5*, *MEFV*, and *WISP3* (Nakano, Boyle, & Firestein, 2013). While these data were not discovered in cells of the immune system, similar results have been reported in PBMCs, for example, hypomethylation of a single CpG motif of the *IL6* gene (Nile, Read, Akil, Duff, & Wilson, 2008), in addition to *IFNGI* promoter hypermethylation in $CD4^{+}CD28^{-}$ T cells (Pieper et al., 2014). Augmented expression of *DNMT1* and *MBD2* has also been discovered in RA patient PBMC samples, with *DNMT1* in particular associated with RA disease activity (Liu et al., 2011). Conversely, a more recent investigation by de Andres et al. (2015) revealed that *DNMT1* expression was attenuated in B cells, T cells, and monocytes, concomitantly with global hypomethylation in the latter two cell types. The alterations in expression of these methylation-regulatory proteins likely contribute to the differential expression of inflammatory cytokines, such as IL-6, and thus the immune cell infiltration into the synovial joints.

1.4.5 Other Diseases

Finally, altered expression of DNMTs in PBMCs has been reported in various other conditions such as Schizophrenia (Auta et al., 2013), Vitiligo (Zhao, Gao, Wu, Tang, & Lu, 2010[b]), Alopecia Areata (Zhao et al., 2012), and Idiopathic Thrombocytopenic Purpura (Tao et al., 2008), in addition to altered global methylation in Alzheimer's disease (Di Francesco et al., 2015) and again, Alopecia Areata (Zhao et al., 2012). Even body weight has been shown to inversely correlate with LINE-1 methylation, suggesting that unhealthy lifestyle factors can cause epigenetic change which may then predispose an individual to disease (Piyathilake, Badiga, Alvarez, Partridge, & Johanning, 2013).

A common underlying theme with the diseases discussed so far is a chronic inflammatory state. As to whether inflammation as a consequence of the disease state then causes faulty epigenetic modifications, or whether these aberrant epigenetic processes are causative of the inflammatory state, thus enhancing susceptibility to a given disease, remains to be fully understood. To add complexity, there is also the possibility that this is a vicious cycle, with inflammation and aberrant epigenetic modifications exacerbating one another. Poor lifestyle habits such as physical inactivity (Abramson & Vaccarino, 2002; Allison, Jensky, Marshall, Bertoni, & Cushman, 2012; Gabel et al., 2015; León-Latre et al., 2014), obesity (Greenberg & Obin, 2006; Zeyda & Stulnig 2009), and smoking (Kanherkar et al., 2014) are all associated with elevated systemic inflammation. Therefore, given the extensively reported reduced incidence, better prognosis, attenuation of fatigue or enhanced quality of life in many of the aforementioned diseases as a consequence of PA (Ayan & Martin, 2007; Colberg et al., 2010; de Jong et al., 2003; Knols, Aaronson, Uebelhart, Fransen, & Aufdemkampe, 2005; Lee, 2003; Loudon, Corrol, Butcher, Rawsthorne, & Bernstein, 1999; McNeely, Campbell, Rowe, Klassen, Mackey, & Courneya, 2006; Segal et al., 2003; Shiroma & Lee, 2010; Singh, Newton, Galvão, Spry, & Baker, 2013; Stenstrom & Minor, 2003), it

seems logical that the anti-inflammatory (Geffken, Cushman, Burke, Polak, Sakkinen, & Tracy, 2001; Gano et al., 2011; Thompson, Markovitch, Betts, Mazzatti, Turner, & Tyrell, 2010), and overall salubrious effects of exercise, would be at least associated with a more favourable epigenetic state. In addition, PBMCs are clearly an interesting population of cells that warrant further examination due to their wide range of immune functions which are able to impact upon many tissues due to their circulation throughout the body.

1.5 *In Vitro* Studies: Inflammation and DNA Methylation

Up to now, DNA methylation changes as a causal factor in the development of chronic inflammation and disease has been discussed. However, the question of whether inflammation in the absence of disease leads to epigenetic modifications should also be addressed. The following literature is summarised in table form in appendix A.

1.5.1 Interleukin 6

Data now suggests that changes in the DNMT enzymes that catalyse changes to the epigenome may indeed be regulated by inflammatory mechanisms. For example, inflammatory bowel disease-associated CRC manifests with high levels of systemic IL-6 (Atreya & Neurath, 2005), while *DNMT1* is also overexpressed in this condition (Foran et al., 2010). *In vitro* studies have shown that IL-6 stimulation of human cancer cell lines (HCT116/K562) resulted in elevated expression and activity of DNMT1 (Foran et al., 2010, Hodge, Xiao, Clausen, Heidecker, Szyf, & Farrar, 2001). This augmentation of DNMT1 activity could occur through PI3K activation of protein kinase B (AKT), and subsequent AKT-dependent phosphorylation of the nuclear localisation signal of DNMT1, allowing nuclear translocation and subsequent binding of DNA (Hodge et al., 2007). PI3K/AKT signalling also regulates *DNMT3B* expression in hepatocellular carcinoma (Mei et al., 2010).

IL-6 has been implicated in the maintenance of promoter methylation in cholangiocarcinoma (Wehbe, Henson, Meng, Mize-Berge, & Patel, 2006) and multiple myeloma cells (Hodge et al., 2005), which is likely to be due to DNMT1, given its functional role. Furthermore, upregulation of *DNMT1*, but not *DNMT3A* or *DNMT3B*, was reported to be responsible for the IL-6 induced hypermethylation of tumour suppressors *p53* and *p21* in A549 (adenocarcinomic human alveolar basal epithelial) cells (Liu et al., 2015[a]). Western blotting showed that the IL-6 mediated increase in *DNMT1* expression was due to activation of the JAK2/STAT3 pathway, which is in support of previous findings that STAT3 induces *DNMT1* expression, while STAT3 depletion downregulates *DNMT1* in malignant T lymphocytes (Zhang, Wang, Woetmann, Raghunath, Odum, & Wasik, 2006). Furthermore, IL-6 induced *DNMT1* mRNA and protein expression correlated with *SOCS3* promoter methylation *via* STAT3 signalling in human colorectal cancer cell cultures (Li, Duering, Peppelenbosch, Kuipers, de Haar, & van der Woude, 2012), which was corroborated by Huang et

al. (2016) who reported that in pancreatic cancer cells, IL-6/STAT3 augmented DNMT activity thereby promoting *SOCS3* promoter hypermethylation. In a novel approach, Braconi et al. (2010) reported similar results to Liu et al. (2015[a]) by transfecting cholangiocarcinoma cell lines with IL-6, and discovered *DNMT1* expression was elevated with a concomitant decrease in tumour suppressor expression. Together, these studies provide a mechanistic pathway between IL-6 and changes in DNA methylation *via* manipulation of DNMT expression or cellular location.

1.5.2 Other Pro-inflammatory Cytokines

IL-1β stimulation of fibroblast-like synoviocytes isolated from RA patients subsequently decreased expression of *DNMT1*, whereas both IL-1β and tumour necrosis factor (TNFα) decreased *DNMT3A* expression (Nakano et al., 2013). In a healthy human lung fibroblast cell line however, greater concentrations IL-1β (10 $ng \cdot ml^{-1}$ compared with 2 $ng \cdot ml^{-1}$ utilised by Nakano et al.) resulted in augmented expression of both *DNMT1* and *DNMT3A* (Huang et al., 2012).

TNFα-induced inhibition of the transmembrane protein Notch-1 has been attributed to an HMT (EZH2) and DNMT3B recruitment *via* NF-κB, resulting in hypermethylation in mouse myoblast cells (Acharyya et al., 2010). TNFα has also been shown to cause non-CpG methylation at the *PGC1α* promoter region in human myotubes (Barrès et al., 2009), while both TNFα and Insuline-like Growth Factor (IGF-1) were able to increase *SOCS3* expression in human coronary artery smooth muscle cells, *via* NK-κB activation and STAT3 phosphorylation, respectively (Dhar, Rakesh, Pankajakshan, & Agrawal, 2013). Interestingly, when these cells were stimulated with TNFα and IGF-1 together, *DNMT1* expression was elevated, in addition to increased promoter methylation and decreased expression of *SOCS3*. These data highlight not only that genetic and epigenetic signatures vastly differ between different cell types, but also that within the same cell populations, combinations of inflammatory mediators can elicit differential effects than when the cytokines were present alone.

1.5.3 Prostaglandin E2

Prostaglandin E_2 (PGE_2), a lipid autocoid derived from arachidonic acid, is an important mediator in the acute inflammatory response, and can regulate *IL6* expression in various cell types (Bagga, Wang, Farias-Eisner, Glaspy, & Reddy, 2003; Hinson, Williams, & Shacter, 1996; Inoue, Takamori, Shimoyama, Ishibashi, Yamamoto, & Koshihara, 2002; Williams & Shacter, 1997).

Expression of *Sp1* and *Sp3* transcription factors, known to regulate DNMT3A (Jinawath, Miyake, Yanagisawa, Akiyama, & Yuasa, 2005), have been shown to be upregulated following PGE_2 stimulation, concomitant with an increase in *DNMT3A* expression in fibroblasts, and a decrease in *DNMT3A* and *DNMT1* expression in RAW macrophages (Huang et al., 2012). Similarly,

pharmacological inhibition of PGE_2 receptors results in attenuated expression of both *DNMT3A* and *DNMT3B* in endometriotic cells (Arosh, Lee, Starzinski-Powitz, & Banu, 2015).

Furthermore, Xia, Wang, Kim, Katoh, and DuBois (2012) reported that compared with healthy human tissue, colorectal carcinomas exhibited a correlation between PGE_2 and *DNMT1* and *DNMT3B* expression. This was replicated *in vitro* with stimulation of various CRC cell lines with PGE_2 leading to increased promoter methylation and decreased expression of *CNR1, MGMT, CDKN2B,* and *MLH1* in the LS-174T cell line and $Apc^{Min/+}$ CRC mouse model.

1.5.4 Caffeine and Ionomycin

Ionomycin, an ionophore that raises intracellular calcium (Ca^{2+}) concentrations, has been used in order to investigate changes to DNA methylation and DNMTs; Laye and Pederson (2010) showed that while 0.1 μM of ionomycin was unable to alter mRNA levels of *DNMT3A* or *DNMT3B* in proliferating myoblasts, it was sufficient to cause a 40% downregulation of both of these enzymes in differentiated human myotubes. Despite utilising a much larger dose of 1 μM in L6 rat myotubes, Barrès et al. (2012) did not report any changes in promoter methylation of their selected genes, although mRNA of *PGC1α, TFAM,* and *MEF2A* was elevated. Interestingly however, 5 mM of caffeine, which is also able to raise intracellular Ca^{2+}, did cause significant promoter region hypomethylation and mRNA upregulation of the three aforementioned genes, in addition to *CS* and *PDK4.*

Lucas et al. (2012) and Barrès et al. (2012) both investigated the effects of *ex vivo* contractions in mouse soleus muscle on gene promoter methylation and subsequent transcription. Both groups reported significant findings; the former found promoter hypomethylation in *PPARδ, PDK4, CS*, and *PGC1α* at 30 minutes and 270 minutes post-contraction, with an increase in mRNA at 180 minutes post-contraction. The latter study also reported promoter hypomethylation of *PPARδ, PDK4,* and *PGC1α,* in addition to *MYOD1* and *MEF2A* at 45 minutes post-contraction, followed by increases in only *PPARδ, PDK4,* and *PGC1α* mRNA at 180 minutes post-contraction.

1.5.5 Antioxidants

All of the literature discussed thus far has addressed either regulation of *de novo* methyltransferases or DNA methylation itself. The literature concerning the use of antioxidant treatment of cells primarily reports the effects on the TET family of enzymes however. 100 $\mu g \cdot ml^{-1}$ of ascorbic acid (AA) was able to increase TET activity in mouse embryonic stem cells, causing a global augmentation of 5-hmC which was lost after three days of AA withdrawal, demonstrating the dynamic nature of epigenetic modifications (Blaschke et al., 2013). Similarly, Minor, Court, Young, and Wang (2013) reported a dose-dependent (0 to 1000 μM) elevation of 5-hmC in mouse embryonic fibroblasts, although changes in *TET* expression could not account for this and 5-hmC

was still found to be elevated in TET-deficient cells. The same group also showed that as low a dose as 10 μM could augment 5-hmC in human HEK-293T and HeLa cell lines. Yin et al. (2013) also reported that *TET1* expression was unaltered, suggesting that AA impacts on the catalysis of *TET* and causes TET-mediated 5-mC oxidation, leading to the dose-dependent increases in 5-hmC and 5-fC, intermediary molecules in the active demethylation process, with a concomitant attenuation of 5-mC.

In contrast to the antioxidant AA, H_2O_2 is often used in order to experimentally induce cellular oxidative stress. Barrès et al. (2012) showed that 1 mM of H_2O_2 was able to cause promoter hypermethylation of *TFAM, MEF2A,* and *CS* in rat L6 myotubes. When combined with data from studies utilising AA, it initially appears that antioxidants have an effect on the demethylation cycle, whereas inducers of oxidative stress have the opposite effect and increase DNA methylation at specific loci.

Not only is exercise known to be associated with augmented concentrations of pro-inflammatory cytokines, particularly following bouts of extended duration (Fischer, 2006; Walsh et al., 2011; Ostrowski, Rohde, Asp, Schjerling, & Pedersen, 1999; Gill et al., 2015), but it has also been shown to be associated with elevated concentrations of plasma PGE_2 (Demers, Harrison, Halbery, & Santen, 1981; Venkatraman, Feng, & Pendergast, 2001), plasma Ca^{2+} (Ljunghall, Joborn, Benson, Fellström, Wide, & Åkerstrom, 1984; Salveson, Johansson, Foxdal, Wide, Piehl-Aulin, & Ljunghall, 1994; Vora, Kukreja, York, Bowser, Hargis, & Williams, 1983), and markers of systemic oxidative stress (Slattery, Bentley, & Coutts, 2015). The *in vitro* research discussed above, therefore, suggests that circulatory changes following exercise, and the subsequent health beneficial effects, may be regulated *via* epigenetic mechanisms.

1.6 Exercise and the DNA Methylome

The following literature is summarised in table form in appendix B.

1.6.1 Rodent Studies

Although not directly applicable to human physiology, rodent models of exercise are often used in order to gain specific mechanistic knowledge which may then inform further research in humans.

1.6.1.1 DNA Methyltransferase Expression and Protein Concentrations

Utilising a freely moving wheel protocol, whereby young (46 day old) mice were either given access to the wheel or remained sedentary for seven days, Abel and Rissman (2013) reported a 14% and 19% attenuation of *DNMT1* mRNA in the hippocampus and cerebellum, respectively. *DNMT3A* and *DNMT3B* expression was also significantly reduced by 17% and 19% within the hippocampus, with no observable change in the cerebellum. These finding were not corroborated

by a study that used ELISAs to measure DNMT1 and DNMT3B protein concentrations within hippocampal brain tissue; three month and 20 month old male Wistar rats exercised on a motorised treadmill at 60% of maximal oxygen uptake ($\dot{V}O_{2max}$) for 20 minutes per day for 14 days. At baseline, older rats exhibited 25% lower DNMT1 protein concentrations compared with younger rats, with no difference in DNMT3B. Following the two week exercise period, there were no significant changes in DNMT1 or DNMT3B concentration in either group. These data seemingly contradict the results of Abel and Rissman's study (2013), suggesting that, despite the extra four days of exercise, the intensity and/or duration of the sessions was not sufficient in order to elicit adaptations in hippocampal protein concentrations of DNMT1 or DNMT3B, regardless of whether transcriptional changes did indeed occur in line with Abel and Rissman's (2013) findings. Acutely however, young rats exhibited a 30% and 45% reduction in DNMT3B and DNMT1 concentrations, one hour post-exercise, which returned to pre-exercise concentrations by 18 hours post-exercise. Rodrigues et al. (2015) also studied a sample of male Wistar rats that swam for 60 minutes per day, five days per week for four weeks, and when combined with restraint stress, a significant reduction was observed in *DNMT1* expression in the frontal cortex compared with the controls. It is impossible to isolate the effects of exercise alone from this data, however, *DNMT1* expression was found to be significantly attenuated in both the hippocampus and hypothalamus, when compared against stress alone (Kashimoto et al., 2016). These results could support the hypothesis that two weeks of exercise may be insufficient to chronically alter protein concentrations of at least DNMT1 in the brain, but longer training periods may elicit an adaptive response.

In line with most *in vivo* and *in vitro* investigations thus far, each tissue appears to possess a unique 'epigenetic profile'. The data discussed above all pertains to the brain, however, skeletal muscle is arguably a more relevant tissue to exercise-specific responses. Carter, Shuen, Joseph, and Hood (2015) utilised a contractile activity stimulus to investigate the *tibialis anterior* muscle of six and 35 month old Fischer 34BN rats, and reported a 1.9-fold elevation in DNMT3B protein. Although an exercise stimulus was not actually used, this result still supports the notion that considerable tissue-specificity exists.

1.6.1.2 Global and Gene-specific DNA Methylation

Investigation of DNA methylation regulatory machinery such as DNMTs is essential in order to elucidate the precise mechanisms that underlie epigenetic modification, however, it is also important to clarify the downstream consequences on DNA methylation itself. Some of the studies discussed in the previous section also measured DNA methylation in addition to DNMT expression and protein concentration. Carter et al. (2015), for example, found a significantly greater proportion of DNA methylation in aged muscle compared to the younger rats, concomitant with 50% lower *PGC1*α transcription at rest. No further details were provided regarding the actual measurement of DNA methylation, and therefore these results must be interpreted with caution. Rodrigues et al.

(2015) reported that exercise and restraint stress did not cause any changes to global methylation in the hypothalamus, frontal cortex, periaquecductal gray, or hippocampus, whereas utilising the same ELISA-based method, Kashimoto et al. (2016) found a significant increase in global methylation in the hypothalamus, demonstrating that exercise alone elicits a drastically different response than exercise and restraint stress combined.

On a gene-specific level, methylation has been quantified in a 230bp region downstream of a *BDNF* promoter in adult male Sprague-Dawley rat hippocampal tissue following seven days of running wheel access. A significant decrease in methylation 148bp upstream of the transcription start site was reported, in addition to 41% and 30% increases in *BDNF* mRNA and protein, respectively (Gomez-Pinillia, Zhuang, Feng, Ying, & Fan, 2011). Kanzleiter et al. (2015) also used a treadmill training protocol using mice, but instead of a targeted approach, built up a methylation and gene expression profile using whole genome sequencing and a whole genome microarray for transcriptional changes. 20 sessions across four weeks elicited a vast number of differentially methylated CpG sites distributed among 2,762 promoter regions. Although over 3,000 genes were differentially expressed following the training period, only 361 were actually associated with differential methylation; 66 hypomethylated genes were associated with augmented expression, and 134 hypermethylated genes associated with attenuated expression. This demonstrates that while changes in methylation can regulate gene expression, the process is not as simple as promoter hypermethylation causes transcriptional downregulation and vice versa. In addition, these data show that many genes, at least in a murine model, may not be regulated by DNA methylation at all, but rather by other mechanisms, including epigenetic and non-epigenetic processes.

The acute epigenetic response to exercise has also been investigated in rodents; following a single 60 minute bout on a rotarod that increased from 35 to 45 rotations per minute, the *quadriceps femoris* muscle of six female mice was removed, with quantitative real-time polymerase chain reaction (qPCR) and TET-assisted bisulfite sequencing (TAB-seq) performed in order to investigate transcriptional and methylation changes in three *PGC1*α transcripts, namely, *PGC1*α*-a, PCG1*α*-b,* and *PGC1*α*-c* which are transcribed from two different promoters (Lochmann, Thomas, Bennett Jr, & Taylor, 2015). At baseline, promoter A which transcribes the *PGC1*α*-a* isoform, was expressed 2.4-fold higher than promoter B, concomitant with significantly lower levels of methylation. Following the acute bout, *PGC1*α*-a* remained unaltered, while *PGC1*α*-b* and *PGC1*α*-c*, transcribed from promoter B, were significantly augmented. These exercise-induced transcriptional changes, however, were not correlated with modifications to DNA. In spite of the small sample size utilised within this study, the use of TAB-seq, which distinguishes between 5-mC and 5-hmC, is a more advanced method for methylation analysis which aids interpretation.

As alluded to within the start of this section, murine models of exercise are useful in order to highlight molecular processes which occur in tissues that are invasive to access such as the brain, or as a foundation on which to build further understanding using humans. The data is strongly

supportive of the posit that exercise can directly cause epigenetic modifications, both to the DNA methylation regulatory machinery and consequently, to DNA itself. The following sections will serve to review all current literature regarding the effects of PA and exercise on DNA methylation in humans.

1.6.2 Human Studies

1.6.2.1 Global DNA Methylation in the Context of Habitual Physical Activity

A number of studies have sought to elucidate the relationship between PA history and measures of global or gene-specific methylation. The association between PA, measured by accelerometry over four days, and global methylation in LINE-1 has been investigated (Zhang et al., 2011). Those who performed approximately 30 minutes of PA per day, when compared to those who performed less than 10 minutes, had a significantly greater level of global DNA methylation in peripheral blood. However, following multivariate adjustment for age, gender, smoking status, ethnicity and body mass index (BMI), the association was no longer statistically significant. Given the methodological limitations of the study it is difficult to draw any firm conclusions, however, it does suggest that habitual PA may be linked with DNA methylation.

More recently, White, Sandler, Bolick, Xu, Taylor, and DeRoo (2013) retrospectively assessed childhood, adolescent, and previous 12 months PA of over 600 non-Hispanic, white women with a family history of breast cancer. The women that reported being active above the median amount for all three periods were shown to have significantly greater peripheral blood LINE-1 methylation than those below the median. Similarly, in an elderly population, Luttropp, Nordfors, Ekström, & Lind (2013) divided 509 individuals aged 70 years and over into four discrete groups based on the amount of light and heavy PA they performed per week, although the timeframe was not specified. Leukocyte global methylation, in this case assessed using the Luminometric Methylation Assay (LUMA) method, was found to be significantly correlated with self-reported activity level, even after adjustment for gender, systolic and diastolic blood pressure (BP), LDL and HDL cholesterol, serum triglycerides, smoking status, and BMI. Despite the cross-sectional nature of the research, and the use of self-reported PA, the data demonstrates that longer term PA could be related to a more favourable epigenetic profile.

1.6.2.2 Gene-specific DNA Methylation in the Context of Habitual Physical Activity

In contrast with the above results which seem to suggest that PA is associated with hypermethylation of DNA across the genome, results from the Commuting Mode and Inflammatory Response Study did not support this association, as PA was not correlated with global (LINE-1) methylation in leukocytes. Interestingly, levels of *IL6* promoter methylation were not significantly associated with any of the study variables, which also included age, gender,

ethnicity and BMI, in addition to PA and diet (Zhang, Santella, Wolff, Kappil, Markowitz, & Morabia, 2012). Specific comparisons between subgroups reiterated this, as neither LINE-1 or *IL6* promoter methylation were different between public transport users and car drivers (Morabia et al., 2012), The substantial age range of the study population (18 to 78 years) is likely to be a significant confounding variable in this instance, given that aging itself is associated with differential DNA methylation (Agrawal, Yang, Agrawal, & Gupta, 2010; Fuke et al., 2004; Heyn et al., 2012). These results were corroborated with a more homogenous, albeit, aged population (Gomes et al., 2012), whilst also using an objective measure of PA in contrast to a block energy expenditure survey as utilised by Zhang et al. (2012). Similarly, in a novel longitudinal study of mixed-sex children (Clarke-Harris et al., 2014), measurements were taken annually from five years of age, until the children were 14 years old. No significant interactions between PA and DNA methylation were reported, however, an interesting and clinically relevant finding was that methylation of four *PGC1*α promoter CpG loci predicted adiposity, as measured by dual-energy X-ray absorptiometry, independently from confounding variables such as gender or PA level.

Most recently as part of the Cardiovascular Health Study, the association between gene-specific methylation changes in PBMCs and PA energy expenditure (PAEE) over eight years was assessed in a sample of elderly men and women (Shaw, Leung, Tapp, Fitzpatrick, Saxton, & Belshaw, 2014). Maintenance of increased PAEE of 500 kcal or more per week resulted in significant hypermethylation of the *TNF* gene, while the *IL10* gene was significantly hypomethylated in those who increased their PAEE by 500 kcal per week, compared with those who decreased their PAEE by the same amount. Given the pro-inflammatory role of TNFα, and the anti-inflammatory role of IL-10, these PAEE-induced modifications appear to represent a favourable outcome. These two studies (Shaw et al., 2014; Zhang et al., 2012) differed drastically from a methodological standpoint; the former measured PA through the use of a questionnaire that selected 26 specific activities performed over the previous year, in contrast to the latter study which involved participants recalling any physical activities over the previous eight years. Furthermore, Shaw et al's (2014) study utilised a much more defined elderly age group in comparison with the Zhang et al's (2012) study that included individuals ranging from 18 to 78 years of age. Interestingly, in a cross-sectional comparison of experienced (defined as three or more years) and novice tai chi practitioners (Ren et al., 2012), six CpG sites of 60 measured across the genome showed differential methylation between the groups, with the more experienced group demonstrating a slowing of the usual age-related pattern of hypomethylation. Despite no specific rationale given for the selection of these specific CpG sites, this result, as well as the fact that age-related hypomethylation has been reported in PBMC samples (Fuke et al., 2004), clearly illustrates how the lack of a properly defined study population can clearly be a large confounding variable, which may account for the lack of significant findings in Zhang et al's study.

1.6.2.3 Disease-specific DNA Methylation in the Context of Habitual Physical Activity

In addition to general gene specific changes discussed above, several studies have attempted to elucidate the epigenetic effects of PA related to disease-specific genetic loci. For example, Coyle, Xie, Lewis, Bu, Milchgrub, and Euhus (2007) utilised a cross-sectional design in order to investigate the effects of self-reported PA on promoter methylation of the tumour suppressor genes *APC* and *RASSF1A*, an epigenetic alteration commonly associated with breast cancer risk. They reported that lifetime, previous five years, and previous year levels of PA were all inversely correlated with promoter methylation of *APC* but not *RASSF1A*, although this association did not reach statistical significance. Similarly, hypermethylation of *APC*, but again, not *RASSF1A*, was inversely associated with the requirement to have breast biopsies. These results appear to suggest that PA may regulate epigenetic modifications in certain tumour suppressor genes, thereby reducing the risk of breast tumour growth. PA has also been shown to be inversely correlated with methylation of *CACNA2D3*, another tumour-suppressor gene, in gastric carcinoma patients (Yuasa et al., 2009) suggesting an anti-tumorigenic effect, although no significant associations were reported for the remaining five tumour-related genes (*CDX2*, *BMP2*, *p16*, *GATA5*, *ER*) that were tested. The data from these two studies shows that PA may convey protective anti-oncogenic effects through modulation of tumour-suppressor methylation. In a large scale study whereby over 1,000 colon cancer patients recalled activity levels for the previous year, in addition to 10 and 20 years ago, no significant correlations were reported between PA and tumour CpG island methylation in *MINT1, MINT2, MINT31, p16,* or *hMLH1* as measured by methylation-specific PCR (Slattery et al., 2007).

Given the relative complexity and weaknesses of measuring PA cross-sectionally, in combination with various cancers which differ substantially on a pathophysiological level depending on the type and location, cross-sectional studies such as the aforementioned are probably insufficient evidence for firmer conclusions. Thus, the role of PA in the methylation status of selected cancer related genes is far from clear and warrants further investigation.

There appears to be a paucity of research investigating the interaction between PA and DNA methylation in diseases other than cancer, although the data that does exist strongly supports the reported associations between PA and disease-related DNA methylation within specific CpG sites. Pirola et al. (2012) found that PA assessed by questionnaire was inversely correlated with *MT-ND6* methylation, but not *MT-CO1* or *D-loop*, in liver mitochondrial DNA samples isolated from non-alcoholic fatty liver patients. Furthermore, a similar association was reported between questionnaire-assessed PA and promoter methylation of *COMT* in peripheral blood samples isolated from schizophrenia patients that possessed the Val/Val genotype (Lott et al., 2013).

From these studies discussed in section 1.6.2 thus far, no firm conclusion can be made regarding the effects of habitual PA on epigenetic modification, however, it is clear that methodologies must be appropriately selected in order to truly quantify any changes that are occurring. Apart from the

issues related to population selection and quantification of PA already discussed, the overall inconsistency in results is likely in part due to the common utilisation of global methylation as an outcome measure, as this does not reflect changes in DNA methylation at the gene-specific level. For example, a wide array of genes may be differentially methylated in response to activity, however, some may be hypomethylated, and others hypermethylated, resulting in little to no global change. Furthermore, while hypomethylation of particular genes is an undesired outcome, hypomethylation and thus transcription of tumour suppressor genes is highly desired in the context of cancer. This highlights the need to investigate gene and CpG sequence-specific changes. Furthermore, the plethora of methods that are currently available to quantify DNA methylation may have also played a role in the inconsistency of the data thus far.

1.6.2.4 Impact of Exercise Training and Physical Activity Interventions on Epigenetic Modification

Over the past six years, a number of well-controlled experimental studies have begun to characterise the effects of exercise and PA interventions on DNA methylation in a number of tissues including skeletal muscle, adipose, tumour biopsies, saliva, sperm, peripheral blood, and leukocytes.

1.6.2.4.1 Skeletal Muscle and Adipose Tissue

Within skeletal muscle, regular exercise elicits a vast number of adaptations attributable to cumulative increases in functional proteins due to transient genetic upregulation (Coffey & Hawley, 2007). Given the role of epigenetic mechanisms in the control of gene transcription, it appears entirely plausible that adaptation to exercise may also involve differential DNA methylation. In support of this, Lindholm et al. (2014) demonstrated that three months of single-leg knee extensions elicited a vast number of epigenetic changes within *vastus lateralis* biopsy samples taken from healthy young men and women. Specifically, the Infinium HumanMethylation 450k array identified 4,919 differentially methylated sites, of which 839 were altered by at least 5% in 4,076 differentially expressed genes. Many of these areas were then validated using bisulfite pyrosequencing. Furthermore, the top enriched categories of molecular function were all linked to muscle structure, function, and bioenergetics, suggesting that epigenetic changes are at least associated with adaptation within the muscle.

These data provide a much more extensive picture of methylation changes within exercising muscle than previous research that investigated the effects of nine days of bed rest, and the subsequent fourweek re-training period using a cycle ergometer on *PGC1α* methylation (Alibegovic et al., 2010). Following the rest period which induced insulin resistance, *vastus lateralis* muscle biopsies taken from the healthy young male participants exhibited significant hypermethylation in one of three CpG sites analysed within the promoter region, however, this did

not fully return to pre-bed rest levels following four weeks of retraining. A concomitant attenuation in *PGC1α* mRNA was discovered following bed rest, which again, did not return to pre-bed rest levels following retraining. Interestingly, these data highlight the rapidity with which physical inactivity can cause relatively persistent epigenetic modifications which may be related to insulin resistance.

T2DM is a complex metabolic disease arising due to a combination of factors including genetic susceptibility and unhealthy lifestyles (Lyssenko & Laakso, 2013). A controlled study in a cohort of individuals with a family history of T2DM indicated that a six-month exercise intervention was sufficient to induce alterations in both global and gene-specific methylation, independent of family history of T2DM (Nitert et al., 2012). Overall, *vastus lateralis* skeletal muscle biopsies showed that hypomethylation occurred in 115 genes, and hypermethylation occurred in 19 genes. Specifically, hypomethylation of *RUNX1* and *MEF2A*, key transcription factors involved in exercise training adaptation (Keller et al., 2011; McGee, Sparling, Olson, & Hargreaves, 2005), *THADA*, associated with T2DM (McCarthy, 2010), and *NDUFC2*, which encodes NADH hydrogenase, the first enzyme of the oxidative phosphorylation system within the mitochondrial inner membrane (Weiss, Friedrich, Hofhaus, & Preis, 1991), were reported following the intervention. Additionally, methylation of *IL7*, which stimulates proliferation of lymphocytes, was decreased and associated with an increase in mRNA expression and serum concentration post-exercise. A separate analysis of the same cohort (Rönn et al., 2013) demonstrated that the exercise intervention decreased abdominal adiposity and diastolic BP, and increased $\dot{V}O_{2max}$, HDL, and adipose tissue global DNA methylation. In addition to the assessment of global methylation, likely performed to enable comparison of results with existing literature, gene-specific analyses were also included, and thus it was confirmed that the intervention indeed facilitated differential CpG site methylation of subcutaneous adipose tissue. The majority of sites were located within gene bodies and intergenic regions of 18 obesity and 21 T2DM candidate genes, such as *ITPR2*, a locus associated with waist-hip ratio (Heid et al., 2010), as well as *KCNQ1* and *TCF712*, which have both been implicated in the pathogenesis of T2DM (McCarthy, 2010; Travers et al., 2013). An inverse relationship between methylation and mRNA expression was observed for *TCF712*, in addition to other candidate genes. Overall, 197 genes showed changes in both methylation level and mRNA expression, with an inverse relationship reported in 58% of these. The extensive analysis of both of these studies adds credence to the hypothesis that exercise causes epigenetic modification that epidemiological studies were previously unable to robustly confirm.

The studies cited thus far have all utilised healthy individuals or those with a family history of disease, and have not compared different exercise modalities. Rowlands et al. (2014) compared 16 weeks of endurance training with the same period of resistance training in a sample of Polynesian adults with T2DM. Analysis using the Infinium Human Methylation 450k array demonstrated that both training modalities elicited global hypomethylation, in addition to a vast number of gene-

specific modifications. For example, *NRF1* promoter hypomethylation occurred which corresponded to the large elevation in *PGC1a* mRNA, highlighting a mechanism by which *NRF1* controls *PGC1α* transcription (Scarpulla, 2002). Furthemore, *FASN* promoter hypermethylation was reported concomitantly with attenuated expression of the transcription factor *SREBP1c*, which has shown to be a regulator of FASN activity in human insulin resistant skeletal muscle, specifically in South Asian populations (Hall et al., 2010). It was suggested that these changes could have then been a causative factor in the reduced intramyocellular lipid concentrations, which may be reflective of changes associated with enhanced insulin sensitivity. Overall, the data demonstrated that endurance activity was associated with augmented mitochondrial enzyme activity and glucose handling due to epigenetic modifications. Resistance training elicited a number of changes to protein levels, albeit to a much lesser degree than endurance training. Upstream CpG island hypomethylation occurred in *GLUT4*, however, skeletal muscle GLUT4 content was not altered. Resistance exercise, therefore, appears to not exert significant health beneficial adaptations to the epigenome in a sample of Type 2 Diabetic adults. In contrast, these data clearly demonstrate that the protective metabolic effects of regular endurance exercise are, at least in part, due to differential DNA methylation in a number of genes that regulate various metabolic processes.

1.6.2.4.2 Whole Blood

Experimental manipulation of exercise mode and intensity has begun to enhance our understanding of how the epigenome of circulating cells responds to prolonged periods of exercise training; a six month training study consisting of high intensity interval walking exercise, utilising an aging sample matched to both aging and young control groups, demonstrated that methylation status of the *p15* tumour suppressor gene was unaffected by exercise or age (Nakajima et al., 2010). However, methylation of the *ASC* gene, involved in IL-1β and IL-18 production, was significantly lower within the elderly population when compared with the young controls, which potentially explains, at least in part, the commonly described age-associated inflammatory state (Chung et al., 2009), and thus, is an important finding within the context of this review. *ASC* methylation of DNA extracted from peripheral blood samples was found to be higher in the older group subjected to the exercise protocol compared with the aging control group, which may indicate that the known anti-inflammatory effect of longer-term moderate exercise may be facilitated *via* attenuation of age-related hypomethylation (Agrawal et al., 2010; Fuke et al., 2004; Heyn et al., 2012). In a much smaller cohort, but utilising the same training stimulus, a genome-wide microarray covering 237,000 probes (Agilent Human CpG Island 244K array) revealed that over 40 genes, including *NFκB2,* were differentially methylated following the six month training period (Zhang et al., 2015), which was validated using pyrosequencing.

These data support the posit that even very moderate intensity exercise programme, when sustained over an extended duration, can possibly reduce chronic low-grade systemic inflammation *via* modification of DNA methylation within inflammatory genes.

1.6.2.4.3 Peripheral Blood Leukocytes

Longer-term moderate exercise has also been reported to have beneficial effects on DNA methylation when employed as remedial or complementary therapy. For example, in primarily sedentary cancer patients, a six-month clinical exercise intervention consisting of 150 minutes per week of moderate intensity aerobic exercise on a treadmill (the control group received only usual clinical care) altered the methylation profile of 43 genes (Zeng et al., 2012). Most profoundly, hypermethylation of *CXCL10*, involved in chemoattraction of monocytes, T cells and NK cells, and *EPS15*, a protein involved in the EGFR pathway, was reported. In addition, hypomethylation of *ABCB1*, a protein involved in cell membrane efflux, *RP11450P7.3*, a gene for a kelch-like family protein, and *KIAA0980*, which encodes ninein-like protein which contributes to chromosome segregation and cytokinesis, was reported. Six of the 43 genes were associated with overall patient survival, with three of these hypomethylated following exercise, suggesting augmented gene expression. One gene was particularly in the context of cancer; *L3MBTL1*, a candidate tumour suppressor gene (Gurvich et al., 2010; Qin et al., 2010), was found to be inversely correlated with gene expression, while there was also an association between low risk of breast cancer death and high levels of expression. Of interest is that this study measured changes in methylation status in peripheral blood leukocyte samples. It has been reported (Reinius et al., 2012) that considerable variation exists between PBMCs and granulocytes, and even within each cell population (T cells, NK cells, B cells, monocytes), a variation that is considerably more pronounced in adult blood than cord blood (Jacoby, Gohrbandt, Clausse, Brons, & Muller, 2012). The investigators did, however, expand on their initial observations by analysing tumour samples, and reported concordance between the two measures in terms of exercise-induced *L3MBTL1* methylation, although blood may not be a useful surrogate for all tissues or tumour samples given the differences in gene-specific methylation reported between muscle, colon, brain, heart, kidney and liver (Kitamura et al., 2007; Pai, Bell, Marioni, Pritchard, & Gilad, 2011).

Not all studies show that exercise training programmes have an effect on DNA methylation although this is more than likely due to a number of methodological issues that have previously been discussed within this literature review, such as the reliance on global methylation as an isolated measure, or differences in mode, intensity, and study population. For example, 12 months of three supervised aerobic sessions per week in a sample of postmenopausal women was insufficient to elicit any significant alterations in global methylation in leukocyte DNA (Duggan, Xiao, Terry, & McTiernan., 2014). It was not elucidated whether any gene-specific changes occurred however, which, given the data presented by the studies discussed thus far, would be

plausible to expect. In support of this, a genome wide analysis revealed a plethora of training-induced changes to the leukocyte methylome following only four weeks of high intensity training (Denham, O'Brien, Marques, & Charchar, 2015[b]). Over 80,000 CpG sites were hypermethylated and over 120,000 hypomethylated, with 19 of these sites exhibiting a 20% or greater change. As would normally be expected from promoter hypomethylation, *UNG* mRNA was then elevated following training. Given the role of UNG in base-excision repair in the active demethylation pathway, this presents a possible mechanism by which exercise is able to cause and maintain epigenomic changes resulting in favourable adaptations. For example, another gene that was hypomethylated in the promoter region, *EGF*, exhibited a concomitant attenuation of mRNA levels, which could be interpreted as beneficial to vascular health considering the role of EGF receptor hyperactivity in atherosclerosis (Makki, Thiel, & Miller Jr, 2013). Small changes in methylation also occurred at various sites in *miRNA21* and *miRNA210*, although the changes observed in the former did not remain significant after the application of a false discovery rate correction. There were, however, significant alterations in mRNA level of both of these miRNAs. Maybe most importantly, the mRNA targets of miRNA-21 included pathways involved in MAPK and Toll-like receptor signalling, and fatty acid metabolism, whereas mRNA targets of miR-210 were pathways related to calcium, B cell receptor, and TGF-β signalling. These data provide a relatively concise mechanistic pathway by which multiple epigenetic modifications work in concert to elicit salubrious exercise-induced adaptations.

1.6.2.4.4 Germ Cells

The same group also studied the transgenerational effects of regular exercise on DNA isolated from sperm. Anecdotal evidence of the effects of environmental exposures on transgenerational inheritance, such as the Dutch Hunger Famine (Heijmans et al., 2008), suggests that epigenetic changes occur in germ cells which are then stable enough to be passed on to the offspring. This has been shown in mice, whereby differentially methylated regions of sperm DNA were passed to the offspring, causing behaviours associated with psychiatric disorders *via* altered gene expression (Milekic et al., 2014). In humans, three months of sprint training of progressive volume, selected to coincide with a single human spermatogenesis cycle, was able to elicit a significant decrease in global methylation quantified by an ELISA-based method (Denham, O'Brien, Harvey, & Charchar, 2015[a]). Further analysis using the Infinium Human Methylation 450k array highlighted a vast number of differentially methylated CpG islands spanning over 4,000 genes. Most importantly within the context of this particular investigation was that 16 paternally imprinted genes associated with various pathologies, including Alzheimer's disease, T2DM, and atherosclerosis, were also differentially methylated following the training period, supporting the concept of environmental stimuli causing significant epigenetic modifications that are then able to be passed on to the next generation, which may then predispose to specific diseases.

1.6.2.4.5 Other Tissues

The use of even more accessible tissues such as saliva to track methylation changes related to disease risk is an interesting and potentially useful screening tool. In the context of cancer risk, Bryan, Magnan, Hooper, Harlaar, and Hutchinson (2013) selected 45 CpG sites that are potentially associated with breast cancer, and investigated the relationship between self-reported PA in addition to objectively measured cardiovascular fitness using a sample of sedentary men and women. The intervention consisted of individually tailored self-help materials, designed to increase PA participation based on the participants' motivational readiness, which, after 12 months, significantly increased time spent exercising, but not $\dot{V}O_{2max}$, when compared with the control group. At baseline, average methylation of the selected CpG sites was inversely correlated with '7 Day Physical Activity Recall' minutes, which remained significant after controlling for age, but not BMI. Following the intervention, the increase in recall score was significantly correlated with a decrease in methylation, even after controlling for age, BMI and baseline $\dot{V}O_{2max}$, highlighting that chronic PA may convey protective effects, even within saliva DNA, that may result in the delay or reversal of aberrant DNA methylation at particular sites which could promote tumorigenesis.

The majority of studies reviewed here indeed agree that a period of four weeks to 12 months is sufficient to modify gene-specific methylation of a number of different genes associated with aging and pathologies such as cancer and T2DM. The significance of this on prognosis or long-term clinical outcome is an important aspect to consider for future investigation, given the potentially far-reaching implications for public health.

1.6.2.5 Effects of an Acute Exercise Bout on the DNA Methylome

The aforementioned studies show that regular exercise is able to exert significant changes to the epigenome, possibly as a mechanism by which exercise-induced adaptations occur. However, in order to fully understand how exercise is able to elicit these changes, it seems logical to investigate the occurrence of changes following a single bout of exercise.

PGC1a receives a lot of research attention due to it's role as a master regulator of mitochondrial biogenesis, and although not directly linked to DNA methylation as such, histone modifiers appear to possess a key role in expression of the gene. Healthy male subjects performed interval cycling at 73, 100 or 133% of peak power output (PPO) and post-exercise changes in gene expression of *PGC1α* and its regulators were assessed in skeletal muscle biopsies (Edgett et al., 2013). Cycling at 100% of PPO augmented *PGC1α* mRNA more than cycling at 73% PPO, but supra-maximal exercise seemed to blunt this response, so that a lower increase in levels of *PGC1α* mRNA was observed when compared to both 100% and 73% PPO. Increases in the mRNA of the regulators *Sirt-1*, *PDK4* and *RIP140* also occurred. The latter, a negative regulator of *PGC1α,* functions as a scaffold protein, linking nuclear receptors to chromatin remodelers such as HDACs (White,

Morganstein, Christian, Seth, Herzog, & Parker, 2008). The finding that *PGC1a* was augmented in an intensity dependent manner following cycling corroborates previous research (Egan et al., 2010). In this study, phosphorylation of a classIIa HDAC was only elevated following a high intensity bout, suggesting a mediating role in *PGC1α* upregulation following exercise. This is of interest in the context of this review due to the close synergistic relationship between HDACs and DNMTs; not only have HDAC1 and HDAC2 been shown to bind with DNMT1 (Fuks, Burgers, Brehm, Hughes-Davies, & Kouzarides, 2000; Rountree, Bachman, & Baylin, 2000), HDAC inhibition also promotes DNMT1 degradation (Arzenani et al., 2011). In addition, in ovarian cancer DNMT3B was shown to correlate with HDAC1 and HDAC2 (Gu et al., 2013). These data highlight a potential pathway by which epigenetic modifications elicit changes to gene transcription, and while these results are only indirectly related to DNA methylation, a recent broad review on epigenetic modulation by exercise (Ntanasis-Stathopoulus, Tzanninis, Philippou, & Koutsilieris, 2013) pointed out that this mechanism may suggest a way by which the hypermethylated status of *PGC1α* in diabetic patients (Barrès et al., 2009) could be beneficially modified.

In a sample of sedentary young men and women following a $\dot{V}O_{2peak}$ test on a cycle ergometer, global methylation of *vastus lateralis* skeletal muscle was reported to be attenuated. Further analysis demonstrated that hypomethylation occurred at promoter regions of *PGC1α*, *PDK4* and *PPARδ* immediately post exercise (Barrès et al., 2012). Consequently, transcription was upregulated, and given the roles of these genes in mitochondrial and lipid metabolism, this would be considered to be a beneficial modification. Manipulation of TET enzymes, responsible for active demethylation, are even less well characterised than DNMTs following exercise, however, it remains to be elucidated whether significant reductions in the *de novo* DNMTs, transcriptional or actual protein activity, would result in DNA hypomethylation as in the case of this study. Bisulfite sequencing, a technique used for validation of DNA methylation, demonstrated that non-CpG sites (CpA, CpT, CpC) comprised the majority of the hypomethylated cytosines in this study. The authors suggested that hydroxylation of the cytosine's methyl group could provide a possible mechanism as to how an acute exercise bout caused demethylation, however, due to poor specificity of the bisulfite technique with regard to distinguishing between methylated and hydroxymethylated cytosines (Huang, Pastor, Shen, Tahiliani, Liu, & Rao, 2010[b]) this mechanism has not yet been clarified. TAB-seq on the other hand, is able to quantify 5-mC and 5-hmC independently from one another (Yu et al., 2012), and thus, may have been a more useful method.

Following three hours of moderate intensity cycling (60% of $\dot{V}O_{2max}$), *DNMT3B*, but not *DNMT3A*, mRNA was decreased by 50% in skeletal muscle samples (Laye & Pedersen, 2010). Despite the large discrepancy between the duration and intensity of the exercise bouts in the two aforementioned studies, if an acute reduction in DNMT availability does indeed result in passive demethylation, the large attenuation of *DNMT3B* could provide a mechanistic explanation of the exercise-induced hypomethylation reported by Barrès et al. (2012).

Contrarily, in a complex study investigating the timing of carbohydrate intake before and after exercise, a significant augmentation of *COX4I1* promoter methylation was reported in the group that consumed the entirety of their carbohydrate intake before the high intensity interval evening session and remained fasted until the 120 minute bout of steady state cycling at 50% of PPO the next morning, while *FABP3* promoter methylation was reported in both experimental groups four hours post-exercise, albeit compared with different timepoints; *COX4I1* was elevated compared with immediately post-exercise, whereas *FABP3* was elevated compared with rest. In spite of these modifications to the methylome, no changes in mRNA of either gene was observed at this point, although *FABP3* mRNA was augmented immediately post-exercise which appears counterintuitive as it would be expected that changes in methylation would precede alterations to transcription (Lane et al., 2015). The fasted group also exhibited elevated promoter methylation at *PPARδ* four hours following the 120 minute bout. The authors suggest that this alteration in promoter methylation may explain the lower levels of mRNA in the fasted group, however, this attenuation was not statistically significant. Rather, the fed group exhibited significantly augmented mRNA levels in comparison. This finding appears contradictory to the current viewpoint that promoter hypermethylation directly reduces transcription but also highlights the complexity of exercise-induced epigenetic modifications. Indeed, the authors do carry on to hypothesise that exercising in a fasted state may prevent the usual *PPARδ* hypomethylation reported by Barrès et al. (2012) due to the lack of glycolytic flux which may well be necessary for such an epigenetic modification to occur. The data concerning the lipid metabolism genes *FABP3* and *PPARδ*, and mitochondrial metabolism gene *COX4I1*, are difficult to interpret prior to further investigation, but do, however, suggest that carbohydrate availability before exercise is able to differentially regulate these genes involved in key metabolic processes.

The contrasting data reported by the three investigations discussed in this section may be explained in part by the differences in study populations; Laye and Pedersen (2010) recruited healthy young men, who were presumably not specifically trained cyclists. In direct contrast, Barrès et al. (2012) utilised sedentary young men and women, whereas Lane et al. (2015) used trained male cyclists. There is likely to be a vastly different acute response between these two populations as a consequence of training-induced adaptations, possibly mediated by epigenetic mechanisms, in the latter group.

In terms of a more general, systemic role of exercise in epigenetic modification, the effect of acute exercise on cells of the immune system has recently been investigated (Robson-Ansley et al., 2014). 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 5km time trial, a protocol previously shown to reliably induce transient elevations in IL-6 (Walshe, Robson-Ansley, St Clair Gibson, Lawrence, Thompson, & Ansley, 2010), was utilised in order to quantify changes in the methylation of PBMCs measured using the now outdated Infinium HumanMethylation 27k

beadchip. Despite no significant alteration in global methylation, an interesting finding was that the exercise-induced increase in plasma IL-6 concentration immediately following the bout was significantly correlated with the methylation status of 11 genes (*SLAMF1, IRAK3, LDB2, TMEM156, FCRL2, CDK9, SIT1, AER61, RAG2, C10orf89, CD40LG*), a number of which are regulators of immune activities. Of particular interest was the effect on *IRAK3*, a key inhibitor of inflammation associated with the metabolic syndrome and obesity. The lack of change in global methylation in this study was also corroborated in NK cells following a half marathon in both cancer survivors and healthy controls (Zimmer et al., 2015).

Given the relatively vast amount of *in vitro* research (section 1.5) demonstrating a fairly robust, albeit cell specific, relationship between inflammatory cytokines and epigenetic regulation, it seems logical to further investigate this in immune cells. While some studies have utilised whole blood or peripheral blood leukocytes (Denham et al., 2015[b]; Luttrop et al., 2013; Nakajima et al., 2010; Shaw et al., 2014; Zhang et al., 2012), PBMCs represent a more homogenous cell population, and are easier to isolate than individual cell types.

1.7 Aims

Although research into the relationship between exercise, inflammation and epigenetic modification is clearly still in its infancy, and the plasticity of the effects seen remains to be established, the reviewed literature (sections 1.5 and 1.6) appears to support the notion that inflammation associated with acute exercise is likely to be a regulatory mechanism of changes in DNA methylation. Furthermore, regular exercise appears to reduce inactivity-associated systemic inflammation, at least in part, by alterations to the methylome, thereby suggesting a cyclic relationship between exercise and epigenetic modification. This opens up an exciting new sub-discipline and could yield information beneficial not only to seemingly healthy individuals, but also to those suffering from a variety of disease states associated with chronic low-grade systemic inflammation.

With the above points in mind, the aims of this research programme, therefore, were to: firstly, characterise the acute changes that occur to the *de novo* DNA methyltransferases DNMT3A and DNMT3B following exercise in PBMCs, and the role of exercise-induced systemic inflammation in this process; secondly, investigate how these changes then translate into functional modifications to the methylome; thirdly, elucidate the role of training status and how this then affects the acute response to a single exercise bout; and finally, to discover whether a longer term training programme in sedentary individuals will manipulate DNA methylation of genes involved in chronic systemic inflammation associated with physical inactivity, and the role of DNMTs in this process.

CHAPTER 2

GENERAL METHODS

2 General Methods

2.1 Ethical Approval

All studies received ethical approval from the Northumbria University Faculty of Health and Life Sciences ethics committee. The study presented in chapter 5 also received appropriate ethical approval from Coventry University Applied Research Committee, given the collaborative nature of the research. All participants were provided with an approved information sheet prior to giving fully informed written consent (appendix C). They were reminded that they could withdraw from the study at any point without discrimination, and upon completion of testing they were provided with a debrief sheet.

2.2 Experimental Procedures

2.2.1 Stature and Mass

Using the International Standards for Anthropometric Assessment (International Society for the Advancement of Kinanthropometry, 2011), stature was measured using a free-standing stadiometer to the nearest 0.1cm (SECA 217, Hamburg, Germany). Participants stood with their feet together and heels, buttocks, and upper part of the back touching the stadiometer. The head was then positioned in the Frankfort plane; the lower edge of the eye socket was positioned by hand so that it was level with the superior notch of the tragus. Participants were instructed to inhale while a gentle upward lift was applied by hand through the mastoid processes, with the top of the stadiometer placed firmly upon the vertex of the skull. Body mass was measured to the nearest 0.1kg using digital scales (SECA 875, Hamburg, Germany) following removal of any excess clothing and footwear, with weight distributed evenly between both feet.

2.2.2 Hip and Waist Circumference

International Standards for Anthropometric Assessment (International Society for the Advancement of Kinanthropometry, 2011) were followed whilst measuring participant hip and waist circumferences. With the participant stood in a relaxed position and arms crossed across the thorax, a flexible anthropometric tape (Hoechstmass Roll Fix measuring tape, Germany) was used to measure the girth of the waist at the narrowest point between the lower costal border and iliac crest. Similarly, the girth of the waist was measured at the greatest posterior protuberance of the buttocks with both feet together. In both cases, it was ensured that the tape was parallel to the floor and not excessively tight as to indent the skin.

2.2.3 Methyl-donor Food Frequency Questionnaire

A methyl-donor food frequency questionnaire (mdFFQ) was administered in chapters 6 and 7 (appendix D). The questionnaire assesses intake of methionine, folate, betaine, and choline which are all involved in the SAM resynthesis cycle outlined in section 1.2.5. The mdFFQ has been reported to be a reliable tool to estimate the average intake of methyl-donors (Pauwels, Doperé, Huybrechts, Godderis, Koppen, & Vansant, 2015), however, the questionnaire has only been validated in a female population thus far.

2.2.4 Venous Blood Sampling

Following thorough hand decontamination, a tourniquet was applied to the arm approximately five finger widths above the antecubital crease. After appropriate site selection of a vein in the antecubital fossa region the area was disinfected with a 70% alcohol wipe. With the bevel of the needle (Safety-Lok, Becton-Dickinson, Oxford, UK) facing upwards, the needle was inserted into the vein at an approximate 30° angle, followed by lithium heparin containing Vacutainer tubes being pushed into the barrel. The tourniquet was released after the second Vacutainer was filled. After all blood was collected, pressure was applied to the puncture site with gauze while the needle was simultaneously withdrawn and immediately disposed of. Participants were instructed not to bend their arm at the elbow in order to reduce the risk of hematoma formation, and were asked to hold the cotton wool on the puncture site until the bleeding had stopped.

2.3 Lab Analyses

2.3.1 Plasma Separation

Immediately following collection of whole blood into Vacutainer tubes they were centrifuged at 1700*g for 10 minutes (Allegra X-22R Centrifuge, Beckman Coulter, California, USA). Plasma was aspirated into 1.5 ml microcentrifuge tubes and immediately stored at -80 ^{o}C.

2.3.2 Plasma Volume Change

Whole blood was used to determine haematocrit and haemoglobin (HemoCue Hb 201+, EKF Diagnostics, Germany), which was subsequently used for calculating shifts in plasma volume (chapters 4 and 5) using Dill and Costill's (1974) guidelines (Alis et al., 2015):

$$Change\ PV(\%) = 100 * ((Hb_{pre}/Hb_{post}) * (100 - Htc_{post})/(100-Htc_{pre})-1)$$

Hb = Haemoglobin; Hct = Haematocrit.

2.3.3 Peripheral Blood Mononuclear Cells - Rationale

As discussed in chapter 1, not only are the genetic and epigenetic profiles of PBMCs linked to various pathologies, peripheral blood cells share 80% of the transcriptome with nine other major tissues (Mohr & Liew, 2007). Furthermore, the muscle biopsy procedure may actually contribute to elevated local inflammation (Malm, 2001), thus it has been postulated that studying the responses of PBMCs to exercise may be a useful alternative (Jiménez-Jiménez et al., 2008). It has also been hypothesised that mononuclear cells, given that they circulate throughout the body and represent systemic changes (Denham et al., 2015[b]), may prove to be an important cell population to investigate in order to link exercise and health (Connolly et al., 2004).

2.3.4 Isolation of Peripheral Blood Mononuclear Cells

15 ml of Lymphoprep (Stemcell Technologies, Vancouver, Canada) was added to LeucoSep centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany), followed by centrifugation for 20 seconds at 400*g. Under a laminar flow hood, whole blood was poured from the Vacutainer tubes into the Leucosep tubes (approximately 20 ml of blood per Leucosep tube). Leucosep tubes were then centrifuged for 12 minutes at 850*g with the brake set to 0. Using sterile pastettes, the top layer of plasma was removed without disturbing the layer of PBMCs which were then poured into a new 50 ml centrifuge tube, which was topped up to 50 ml with R0 medium (RPMI 1640 medium plus Penicillin/Streptomycin/Glutamine, Sigma Aldrich, Missouri, USA). These tubes were centrifuged for 10 minutes at 400*g. The supernatant was poured off and the cell pellet resuspended by tapping the tube. A freezing medium was prepared of nine-parts fetal calf serum (FCS) to one-part Dimethyl sulfoxide (DMSO), added to the cells, transferred into microtubes and placed into a Mr Frosty freezing container (Sigma Aldrich, Missouri, USA) to be stored at -80 ^{o}C.

2.3.5 Exercise-conditioned Plasma Stimulation of Peripheral Blood Mononuclear Cells

Exercise is well known to elicit a vast number of changes to the proteins and molecules located in the plasma, including, but not limited to, changes in pro- and anti-inflammatory cytokines. In order to elucidate the role of circulating proteins on DNA methylation and DNMTs, it was necessary to control for exercise-induced changes to the fractional cellular composition of PBMC samples (Connolly et al., 2004) by utilising an identical cell sample isolated from a single, resting participant in all experimental conditions in each study. Furthermore, the existence of single nucleotide polymorphisms of genes involved in the DNA methylation cycle has led to the assertion that some individuals may exhibit a genetic susceptibility to epigenetic modification (Terruzzi et al., 2011) and therefore a standardised set of cells was essential to be able to isolate the specific effects of exercise-conditioned plasma. It has been demonstrated that exposure to blood from young mice can aid in muscle and skin healing in older mice due to proteins and RNA located in

the plasma, and red or white blood cells (Conboy, Conboy, Wagers, Girma, Weissman, & Rando, 2005), suggesting that plasma alone can exert a significant impact on various cellular signals. This supports the use of our experimental protocol to clarify the role of circulating proteins on epigenetic modification in PBMCs.

In all chapters, the following procedure was used prior to further analysis. PBMC samples were thawed quickly in a waterbath at 37ºC, transferred to a 15 ml tube containing 10 ml of R0 medium and centrifuged at 400*g for five minutes. Supernatants were discarded and cells were counted using a Casey TT cell counter and analyser system (Roche, Basel, Switzerland). Cells were then diluted accordingly with R0 medium in order to yield $4*10^6$ cells·ml^{-1}. In order to extract nuclear proteins (section 2.3.6), on a 24 well cell culture plate (Cellstar, Greiner Bio One, Gloucestershire, UK) 250 µl of PBMCs ($1*10^6$ cells) were stimulated with 100 µl of plasma separated from whole blood of the exercising participants, 150 µl of R0 medium, followed by incubation at 37 ºC for four hours. The utilisation of an incubation period of this duration was to mimic the post-exercise state in which transient elevations of circulating IL-6 may be maintained for up to four hours; following a marathon, Toft et al. (2000) and Ostrowski et al. (1999) both reported that plasma IL-6 remained elevated for three and four hours respectively. Various other studies have shown post-exercise elevations immediately following cessation of exercise (Bernecker, Scherr, Schinner, Braun, Scherbaum, & Halle, 2013; Nieman et al., 2001), but did not take further measurements until 24 hours (Howatson et al., 2010; Scherr et al., 2011). Therefore, given the duration and relative intensity of the exercise bouts utilised within the forthcoming chapters, it was decided that four hours was a sensible and informed choice of incubation period.

In chapters 3 to 6, PBMCs isolated from a single resting participant were used in all experimental conditions, however, chapter 7 investigates both acute and chronic changes, therefore it was deemed necessary to use cells that had been isolated from the blood of sedentary individuals prior to the training programme, but also cells isolated after exercise-induced adaptations had occurred.

2.3.6 Nuclear Extraction

Cells were transferred into a 15 ml conical tube and centrifuged for five minutes at 400*g. The supernatant was discarded and the cells washing with 500 µL of 1x phosphate buffered saline (PBS) by centrifugation for five minutes at 300*g. The supernatant was again discarded, and cells resuspended by tapping of the tube. A 10x pre-extraction buffer was diluted with distilled water at a 1:10 ratio, followed by the addition of Diothiothreitol (DTT) solution and Protease Inhibitor Cocktail (PIC) to the 1x pre-extraction buffer at a 1:1000 ratio. 100 µL of the complete 1x pre-extraction buffer was added per 10^6 cells, and transferred to a microcentrifuge vial. Vials were then incubated on ice for 10 minutes, vortexed vigorously for 10 seconds, followed by centrifugation for 1 minute at 12,000 rotations per minute in a microcentrifuge. The cytoplasmic extract was carefully removed from the nuclear pellet using a pipette and sterile tips, and stored at -80ºC for later

analysis (chapter 6 only). DTT and PIC were then added to the extraction buffer at a 1:1000 ratio, and 20 µL per 10^6 cells was added. The extract was then incubated on ice for 15 minutes with vortexing for five seconds every three minutes. The suspension was then centrifuged for 10 minutes at rotations per minute in a microcentrifuge, and the supernatant transferred to new clean microcentrifuge vials.

2.3.7 Bradford Assay

A Bradford assay was used in order to quantify the amount of protein per µl of nuclear extract (Quick Start Bradford Protein Assay, Bio Rad, California, USA). 5 µl of each of the seven Bovine Gamma-Globulin standards (2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 $mg \cdot ml^{-1}$), and 5 µl of each of the nuclear extracts, were added to duplicate wells. Each well then received 250 µl of 1x dye reagent (containing methanol and phosphoric acid). The microplate was placed on a microplate mixer at 120 rotations per minute for 10 minutes at room temperature, then read using a microplate reader (Synergy HT, Bio Tek, Vermont, USA) at an absorbance of 595 nm. The average blank value was subtracted from the sample absorbance, and a standard curve was generated in order to quantify protein amount for each nuclear extract.

2.3.8 DNA Methyltransferase ELISA

Quantification of nuclear DNMT3A and DNMT3B concentrations were performed as per manufacturer guidelines (Epiquik DNMT3A/B Assay Kit, Epigentek, New York, USA). Briefly, nuclear extracts were loaded into well in duplicate and incubated with the capture reagent and assay buffer. After washing the wells, an affinity antibody was added, and following further washing, a detection antibody added. Microplates were scanned at 450 nm with a reference wavelength of 655 nm to measure absorbance following the addition of a colour developing solution (Synergy HT, BioTek, Winooski, USA). A standard curve was generated from diluted DNMT3A/DNMT3B standards ranging from 1 $ng \cdot \mu l^{-1}$ to 20 $ng \cdot \mu l^{-1}$. The following equation was then used to quantify enzyme concentration:

*Concentration (ng·mg of protein^{-1}) = (sample OD – blank OD)/(slope*protein amount)*1000*

OD is optical density/absorbance; Slope generated from the standard curve; Protein amount (µg) as quantified by Bradford assay.

2.3.9 Interleukin-6 ELISA

A QuantiGlo Human IL-6 ELISA (R&D Systems, Minneapolis, USA) was used in order to quantify pre- and post-exercise plasma concentrations of IL-6 as per standard manufacturer guidelines. Samples were assayed in duplicate, including positive controls and various dilutions (1500, 300, 60, 12, 2.4, 0.48 $pg \cdot ml^{-1}$) of the IL-6 standard which were used to plot a standard curve.

The mean minimum detectable dose was 0.16 pg·ml-1. Assay sensitivity was 0.35 pg·ml^{-1}, and the assay detection range was between 0.480 and 1,500 pg·ml^{-1}.

CHAPTER 3

PILOT INVESTIGATIONS

3 Pilot Investigations

3.1 Introduction

As outlined in chapter 2, stimulation of PBMCs with exercise-conditioned plasma is a new technique designed to mimic the effects of circulatory changes following exercise in a controlled manner, therefore, it was important to ensure that this process did not result in a significant reduction in cell viability prior to further analysis.

Furthermore, a large body of evidence (section 1.5) demonstrates that pro-inflammatory cytokines can elicit significant changes to the transcription and cellular localisation of DNA methylation-regulatory enzymes *in vitro*. Given Robson-Ansley et al's finding that post-exercise elevations in plasma IL-6 were associated with methylation at a number of CpG loci in PBMCs, combined with this *in vitro* data, a logical assertion would be that IL-6 may be an important regulator of DNMTs following exercise, resulting in exercise-induced epigenetic modifications. Thus far however, no such experimental data has been reported concerning the direct effects of inflammatory cytokines on DNMT transcription or localisation in PBMCs, nor has IL-6 been shown to exert significant influence over the *de novo* DNA methyltransferases DNMT3A and DNMT3B.

The aim of this initial investigation, therefore, was to initially examine whether exercise-conditioned plasma significantly affected PBMC viability following the incubation period, and to quantify whether recombinant IL-6 (rIL-6) was able to significantly alter nuclear concentrations of DNMT3A and DNMT3B *in vitro*. Furthermore, since this is a very contemporary area of research, particularly within exercise physiology, these initial investigations also serve to provide some data on which sample size and statistical power calculations can be based. Based upon the literature discussed in section 1.5.1, it was hypothesized that rIL-6 would cause an augmentation of DNMT3A and DNMT3B nuclear concentrations.

3.2 Methods

3.2.1 Peripheral Blood Mononuclear Cell Viability

Preliminarily, PBMC viability following incubation with exercise-conditioned plasma was calculated. Whole blood was collected from a rested recreationally active male into six 10 ml lithium heparin vacutainer tubes (Becton Dickinson, Oxford, UK). 'Recreationally active' in this instance was defined as currently engaging in exercise for at least the minimum government guidelines of 150 minutes of moderate intensity exercise per week, but not currently involved in intense sport-specific training. PBMCs were isolated using centrifugation of a LeucoSep centrifuge tube (Greiner Bio-One, Frickenhausen, Germany) containing Lymphoprep (Stemcell Technologies, Vancouver, Canada) as outlined in chapter 2. Following counting of the cells, they were diluted accordingly in order to yield $4*10^6$ cells·ml^{-1}. 250 µl of PBMCs ($1*10^6$ cells) were treated with 100 µl of either pre-exercise plasma, post-exercise plasma (exercise protocol outlined in section 4.2), or fetal calf serum (FCS), in addition to 150 µl of RPMI-1640 medium and Penicillin/Streptomycin/Glutamine solution (Sigma Aldrich, Missouri, USA),followed by incubation at 37 ^{o}C for four hours. Each condition was performed in triplicate. Before and after incubation, the cells were mixed with Trypan Blue solution (Sigma Aldrich, Missouri, USA) in a 1:1 ratio, followed by 10 µl of the resultant solution being pipetted into the upper chamber of an Improved Neubauer cell counting chamber (Hawksley, Sussex, United Kingdom). The number of live and dead cells was then counted using a Nikon TMS-F inverted microscope (Nikon, Tokyo, Japan).

3.2.2 Recombinant IL-6 Stimulation

In order to trial nuclear extraction and DNMT ELISA protocols, a preliminary experiment was performed whereby 250 µl of PBMCs ($1*10^6$ cells) were treated with 150 µl of R0 medium containing 10 and 100 ng·ml^{-1} of rIL-6 and 100 µl of FCS followed by incubation at 37 ^{o}C for four hours. 100 ng·ml^{-1} has previously been shown (Foran et al., 2010; Hodge et al., 2005) to increase *DNMT1* expression in a multiple myeloma cell line, while 10 ng·ml^{-1} was utilised by Liu et al. (2015[a]), who reported an increase in *DNMT1* expression in a lung cancer cell line. Nuclear protein extraction was then performed as per the manufacturer guidelines and nuclear DNMT3A and DNMT3B protein concentrations quantified, as outlined in sections 2.3.6 and 2.3.7.

Although the concentrations of rIL-6 that were utilised mimicked previous research, albeit using different cell lines, these would be considered to be supra-physiological and not representative of pre- or post-exercise plasma concentrations. Therefore, in a separate experiment, 250 µl of PBMCs ($1*10^6$ cells) were treated with 150 µl of R0 medium (section 2.3.5) containing various concentrations of rIL-6: 0, 0.01, 0.1, 1, 10, and 100 ng·ml^{-1} followed by incubation at 37 ^{o}C for four hours. The lowest concentration would be considered to be within the expected physiological range

following prolonged endurance exercise (i.e. 10 pg·ml^{-1}). In both rIL-6 experiments, each condition was performed in duplicate. Intra-assay coefficient of variations was calculated as 9.8% and 10.8% for DNMT3A and DNMT3B, respectively.

3.2.3 Statistical Analysis

Data was analysed using IBM SPSS Statistics version 22. Normality of data was ensured using the Shapiro-Wilk test of normality. Non-normally distributed data was subsequently $\log_{10}$ transformed. Paired Samples T Tests were used to test for significant differences between pre- and post-incubation cell viability, and nuclear concentrations following the first rIL-6 experiment. As the subsequent experiment involved a larger range of rIL-6 concentrations, Mauchly's test of sphericity and a one-way repeated measures analysis of variance (ANOVA) was utilised. Levene's Test for Equality of Variances and Independent Samples T Tests were used to analyse differences between enzymes at each concentration of rIL-6. Statistical significance was defined as $p \leq 0.05$.

3.3 Results

3.3.1 Peripheral Blood Mononuclear Cell Viability

Table 3.1 shows that in each condition, there was no statistically significant difference in cell viability before and after incubation for four hours at 37 °C.

Table 3.1 *- Mean (± SD) percentage of viable peripheral blood mononuclear cells before and after incubation period.*

Condition	Mean (± SD) Pre-incubation (%)	Mean (± SD) Post-incubation (%)
Pre-exercise plasma	99.2 (± 0.7)	98.1 (± 0.1)
Post-exercise plasma	98.7 (± 0.1)	96.9 (± 2.7)
Fetal calf serum	95.6 (± 1.5)	97.7 (± 2.0)

3.3.2 Recombinant IL-6 Stimulation

Table 3.2 shows that as the dose of rIL-6 increases, nuclear concentrations of both DNMT3A and DNMT3B also significantly increase.

Table 3.2 *- Mean (± SD) peripheral blood mononuclear cell nuclear concentration of DNMT3A and DNMT3B following stimulation with recombinant interleukin 6 (10 and 100 ng·ml^{-1}).*

rIL-6 Concentration (ng·ml^{-1})	Mean (± SD) Nuclear DNMT3A Concentration (ng·mg protein^{-1})	Mean (± SD) Nuclear DNMT3B Concentration (ng·mg protein^{-1})
10	2.0 (± 1.3)	365.6 (± 11.4)
100	8.8 (± 3.4)[a]	490.5 (± 90.2)[a]

[a]significantly greater than '10 ng·ml^{-1}' ($p < 0.05$).

Table 3.3 represents a larger range of rIL-6 concentrations, ranging from normal physiological ranges up to supra-physiological concentrations. Every concentration of rIL-6 significantly augments nuclear DNMT3A concentrations compared with the blank control. 1 ng·ml^{-1} of rIL-6 also caused a significant increase compared with 0.01 ng·ml^{-1}, however, no other significant effects were observed.

With regard to DNMT3B, 0.01 ng·ml^{-1} and 1 ng·ml^{-1} of rIL-6 did not exert a statistically significant effect. 0.1 ng·ml^{-1} was found to be significantly augmented compared with the blank control, while the two supra-physiological concentrations that had previously been utilised, significantly elevated nuclear DNMT3B concentrations compared with every other rIL-6 condition.

Furthermore, nuclear DNMT3B concentrations were significantly greater than nuclear DNMT3A concentrations in the following conditions: 0 (p = 0.001), 0.1 (p = 0.018), 10 (p = 0.019), and 100 $ng \cdot ml^{-1}$ (p = 0.028).

Table 3.3 *- Mean (± SD) peripheral blood mononuclear cell nuclear concentration of DNMT3A and DNMT3B following stimulation with various concentrations of recombinant interleukin 6 (rIL-6) (0, 0.01, 0.1, 1, 10, 100 $ng \cdot ml^{-1}$).*

rIL-6 Concentration ($ng \cdot ml^{-1}$)	**Mean (± SD) Nuclear DNMT3A Concentration ($ng \cdot mg\ protein^{-1}$)**	**Mean (± SD) Nuclear DNMT3B Concentration ($ng \cdot mg\ protein^{-1}$)**
0	3.3 (± 0.9)	32.8 (± 0.8)
0.01	28.9 (± 3.2)[a]	46.1 (± 16.7)
0.1	44.2 (± 1.1)[a]	88.8 (± 3.2)[a]
1	53.0 (± 4.5)[ab]	90.9 (± 11.3)
10	60.3 (± 1.6)[a]	205.1 (± 7.7)[abcd]
100	80.2 (± 5.7)[a]	458.7 (± 28.7)[abcde]

[a]significantly greater than '0 $ng \cdot ml^{-1}$' ($p < 0.05$); [b]significantly greater than '0.01 $ng \cdot ml^{-1}$' ($p < 0.05$); [c]significantly greater than '0.1 $ng \cdot ml^{-1}$' ($p < 0.05$); [d]significantly greater than '1 $ng \cdot ml^{-1}$' ($p < 0.05$); [e]significantly greater than '10 $ng \cdot ml^{-1}$' ($p < 0.05$).

3.4 Discussion

The results of the initial pilot investigation presented within this chapter demonstrate that incubating PBMCs in the presence of exercise-conditioned plasma does not alter cell viability, even compared with the commonly utilised addition of FCS during cell culture. This procedure, therefore, is suitable for future investigation.

This is also the first known study using human PBMCs to mimic previous *in vitro* data that has consistently demonstrated that when cells are cultured in the presence of rIL-6, expression of DNMTs can be significantly altered (section 1.5.1). A novel aspect of this present study is that nuclear protein concentrations of the *de novo* DNMTs were measured, theoretically representing methylating capacity due to nuclear protein abundance. Furthermore, as most studies have focussed on disease-specific cell lines, supra-physiological concentrations of rIL-6 are often utilised. Since the focus of the research presented in this thesis is exercise-induced modifications in healthy males, it seemed a logical progression to also test normal physiological ranges of rIL-6; 0.01 and 0.1 $ng{\cdot}ml^{-1}$ convert to 10 and 100 $pg{\cdot}ml^{-1}$ – typical post-exercise plasma IL-6 concentrations, dependent upon duration and intensity (Fischer, 2006).

Although the pathways by which IL-6 augments DNMT activity have not been fully elucidated, some results suggest PI3K-dependent activation of AKT and subsequent phosphorylation of the DNMT nuclear localisation signal may cause cellular translocation from the cytoplasm into the nucleus (Hodge et al., 2007). Since IL-6 has been shown to target PI3K in a variety of cell types (Hideshima, Nakamura, Chauhan, Anderson, 2001; Smith, Kiba, Zong, & Witte, 2013; Wegiel, Bjartell, Culig, & Persson, 2008; Xie et al., 2004; Yang et al., 2003), this appears to be a likely signalling pathway by which IL-6 is able to alter nuclear concentrations of DNMTs.

Due to limited resources and the fact that these experiments were pilot investigations on which further studies would potentially be based, a small number of datapoints were used for statistical testing, potentially resulting in type 2 errors. Visually however, the data appears to suggest a linear dose-response relationship between rIL-6 and nuclear DNMT3A, and an almost exponential elevation in DNMT3B with increasing rIL-6 concentrations.

3.4.1 Conclusion

In conclusion, the data strongly supports the hypothesis that IL-6 is able to significantly alter nuclear concentrations of the *de novo* DNA methyltransferases DNMT3A and DNMT3B, and warrants further investigation into whether post-exercise plasma IL-6 may be a significant regulator of exercise-induced epigenetic modifications via manipulation of DNMT nuclear concentration.

CHAPTER 4

THE EFFECTS OF EXERCISE-CONDITIONED PLASMA ON NUCLEAR CONCENTRATIONS OF DNMT3A AND DNMT3B FOLLOWING AN ACUTE BOUT OF INTENSE AEROBIC EXERCISE

4 The Effects of Exercise-Conditioned Plasma on Nuclear Concentrations of DNMT3A and DNMT3B Following an Acute Bout of Intense Aerobic Exercise

4.1 Introduction

In the previous chapter, pilot data corroborated *in vitro* studies that have demonstrated that IL-6 is able to significantly affect nuclear localisation of DNMTs and supports the rationale that exercise-induced changes in DNA methylation may be regulated through IL-6 dependent pathways.

A number of studies have shown that long term exercise, in the form of habitual PA or exercise intervention, is able to elicit changes to the global and gene-specific methylome. Much less is known regarding the acute effects, which may aid in the understanding of the underlying processes by which longer term adaptations occur. Barrès et al. (2012) reported that a single $\dot{V}O_{2peak}$ test conducted on a cycle ergometer was able to cause global hypomethylation of *vastus lateralis* skeletal muscle in a cohort of sedentary young men and women. Hypomethylation occurred at the *PGC-1α, PDK4, PPARδ, TFAM,* and *CS g*ene promoter regions concomitantly with transcriptional upregulation. The methylation status of muscle specific genes *MEF2A* and *MYOD1* remained unaltered. This suggests that the exercise-induced upregulation (mRNA and protein level) of genes involved in mitochondrial biogenesis and substrate metabolism (Coffey & Hawley, 2007) is due to epigenetic modification. However, there is very little data to explain the underlying molecular mechanisms by which these changes occur. Furthermore, Robson-Ansley et al. (2014) reported that plasma IL-6 may be linked with DNA methylation in human PBMCs. The researchers investigated the effect 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 (previously shown to induce elevations in plasma IL-6 – (Walshe et al., 2010)), followed by a 5 km time trial, on subsequent changes in DNA methylation as measured by the Infinium HumanMethylation 27k beadchip. Despite no significant change in global DNA methylation, the exercise-induced elevation in plasma IL-6 concentration was significantly correlated with the methylation status of 11 genes (*SLAMF1, IRAK3, LDB2, TMEM156, FCRL2, CDK9, SIT1, AER61, RAG2, C10orf89, CD40LG*), a number of which are regulators of activities involving B and T cells, while *IRAK3* is a key inhibitor of inflammation associated with the metabolic syndrome and obesity. Given the *in vitro* data discussed in section 1.5 and our pilot investigation data, it seems likely that IL-6 plays a key role in modifying DNA methylation via manipulation of DNMT expression and/or cellular translocation, and therefore, warrants further investigation.

The aim of the present study was to characterise potential changes in PBMC nuclear concentrations of the *de novo* DNA methyltransferases DNMT3A and DNMT3B caused by circulatory factors located in the plasma following a protocol shown to elicit an acute, transient increase in plasma IL-

6. It was hypothesized that nuclear concentrations of DNMT3A and DNMT3B would be significantly attenuated following stimulation with post-exercise plasma, in line with results reported by Laye and Pedersen (2010), who demonstrated that *DNMT3B* transcription was reduced post-exercise, albeit in skeletal muscle samples.

4.2 Methods

4.2.1 Participants

10 recreationally active males were recruited from Northumbria University to take part in the exercise trial. ‘Recreationally active’ was defined as regularly meeting the government’s exercise guidelines of 150 minutes of moderate intensity activity per week, but not currently undertaking vigorous resistance or plyometric training more than once per week. Mean (± SD) characteristics are reported in table 4.1. Participants gave written informed consent, and all methods were approved by the Northumbria University Ethics Committee. All PBMCs were isolated from a resting, recreationally active participant who was recruited under the same criteria as other participants. Furthermore, his characteristics fit within the standard deviation of the exercising participants’ age, height, weight and BMI.

4.2.1.1 Sample Size Estimation

At the time of planning of this study, there had not been any data published that reported changes in nuclear concentrations in DNA methyltransferase enzymes, and therefore, power calculations were based upon initial findings presented in chapter 3 that recombinant human IL-6 was able to significantly augment nuclear concentrations of these enzymes (appendix E). Sample size was calculated using a spreadsheet accessed from the Boston University and Boston Medical Centre Research Compliance website (“Sample-Size Calculations”, 2014).

4.2.2 Experimental Protocol

The following protocol has previously been reported elsewhere (Walshe et al., 2010) and has been shown to elicit a significant transient increase in plasma IL-6. The protocol is described in full in section 4.2.2.3.

4.2.2.1 Familiarisation

Participants first attended the lab in order to be familiarised with the study protocol. This involved completing the full experimental protocol, as described below, without blood samples being taken.

4.2.2.2 $\dot{V}O_{2max}$ Assessment

Seven days after the initial familiarisation, $\dot{V}O_{2max}$ was quantified using a motorised treadmill (Pulsar, h/p/cosmos, Germany). Treadmill speed was set to 12 km·hour^{-1} on a 1% gradient, and increased by 1 km·hour^{-1} every three minutes until the participant reached volitional exhaustion. An online breath by breath analyser (Metalyzer 3B, Cortex, Germany) measured inspired and expired gases, which was used to calculate running velocity at $\dot{V}O_{2max}$ (v$\dot{V}O_{2max}$). Heart rate (HR) was

recorded via short range telemetry (Polar RS400, Finland) during the last 30 seconds of each stage. The test was considered maximal if two of the following criteria were met; a HR greater than 90% of the age predicted maximum (220 beats·min^{-1} – age), a change in $\dot{V}O_2$ of less than 2 ml·kg^{-1}·min^{-1} in the final two stages, or a respiratory exchange ratio of 1.15 or greater.

4.2.2.3 Main Trial

The main experimental trial was conducted following a rest period of seven days. Participants were instructed to abstain from exercise, alcohol and caffeine for the 24 hour period prior to testing. Participants arrived at 09:00 following an overnight 12 hour fast, during which they were allowed to consume water *ad libitum*. Stature and mass were measured using standard procedures (section 2.2.1), and 22.5 ml of blood was then collected into Lithium Heparin containing vacutainers (Becton Dickinson, Oxford, UK) from a vein in the antecubital fossa of the forearm (section 2.2.4). The main protocol required participants to run at 60% of v$\dot{V}O_{2max}$ for 120 minutes, interspersed with sprints at 90% of v$\dot{V}O_{2max}$ for the last 30 seconds of every 10 minutes. Immediately upon completion of the run, a further blood sample was taken using the same procedure as previously described. Pre- and post-run blood samples were centrifuged at 1700*g for 10 minutes at 4 ^{o}C in order for plasma to be separated. Samples were immediately stored at -80 ^{o}C for later analysis. Plasma volume shift was determined as described in section 2.3.2.

4.2.3 Lab Analyses

As described in full in sections 2.3.4 and 2.3.5, a standardised sample of PBMCs were incubated with exercise-conditioned plasma isolated before and after exercise. Nuclear proteins were then extracted and quantified by Bradford assay prior to analysis of DNMT quantification.

4.2.3.1 DNMT Quantification

Quantification of nuclear DNMT3A and DNMT3B enzyme concentrations were performed as per manufacturer guidelines described in section 2.3.8 (Epiquik DNMT3A/B Assay Kit, Epigentek, New York, USA). All samples were assayed in duplicate. Intra-assay coefficient of variation for DNMT3A and DNMT3B was calculated as 3.95% and 9.92%, respectively.

4.2.3.2 Plasma Interleukin-6 Assay

As outlined in section 2.3.9, a QuantiGlo Human IL-6 ELISA (R&D Systems, Minneapolis, USA) was used in order to quantify pre- and post-exercise plasma concentrations of IL-6 as per standard manufacturer guidelines. Intra-assay coefficient of variation was calculated as 4.3%.

4.2.4 Statistical Analysis

Data was analysed using IBM SPSS Statistics version 22. Prior to performing parametric testing of the data, the Shapiro-Wilk test of normality was utilised. Non-normally distributed data was subsequently $\log_{10}$ transformed. Paired Samples T Tests were used to test for significance of repeated measures data. For independent group analysis, Levene's test was utilised in order to ensure equality of variances, followed by Independent Samples T Test. Levene's Test for Equality of Variances and an Independent Samples T Test were used to analyse differences between enzymes at baseline. Statistical significance was set as $p \leq 0.05$.

4.3 Results

4.3.1 Participant Characteristics

Mean characteristics of the participants are provided in Table 4.1. Mean BMI shows that participants were within the normal healthy range of less than 25 kg·m^2. Participants were defined as recreationally active and showed a relatively high level of aerobic fitness, reflected by a mean maximal oxygen consumption of approximately 51.9 ml·kg^{-1}·min^{-1}.

Table 4.1 *- Mean (± SD) participant characteristics at baseline (n = 10).*

Variable	**Mean (± SD)**
Age (years)	24 (± 3)
Stature (cm)	178.5 (± 5.6)
Mass (kg)	77.5 (± 8.8)
Body Mass Index (kg·m^2)	24.3 (± 2.6)
$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	51.9 (± 6.7)

4.3.2 DNMT Concentrations

The effects of cell stimulation with plasma isolated following 120 minutes of treadmill running on subsequent changes in nuclear concentrations of the *de novo* DNA methyltransferases DNMT3A and DNMT3B are illustrated visually in figures 4.1 and 4.2. No change was observed in nuclear concentration of DNMT3A following the exercise stimulus, with mean (± SD) 'pre' and 'post' concentrations measured at 43.1 (± 8.7) and 41.5 (± 17.4) ng·mg protein^{-1} respectively.

Conversely however, mean (± SD) nuclear concentrations of DNMT3B significantly decreased from 365.9 (± 363.6) to 87.2 (± 73.3) ng·mg protein^{-1} (p = 0.042); an approximate 76% reduction immediately following the exercise bout.

Baseline concentrations of DNMT3B were significantly higher than DNMT3A (p = 0.02), showing that DNMT3B is more immediately abundant within PBMC nuclei, corroborating data presented in chapter 3.

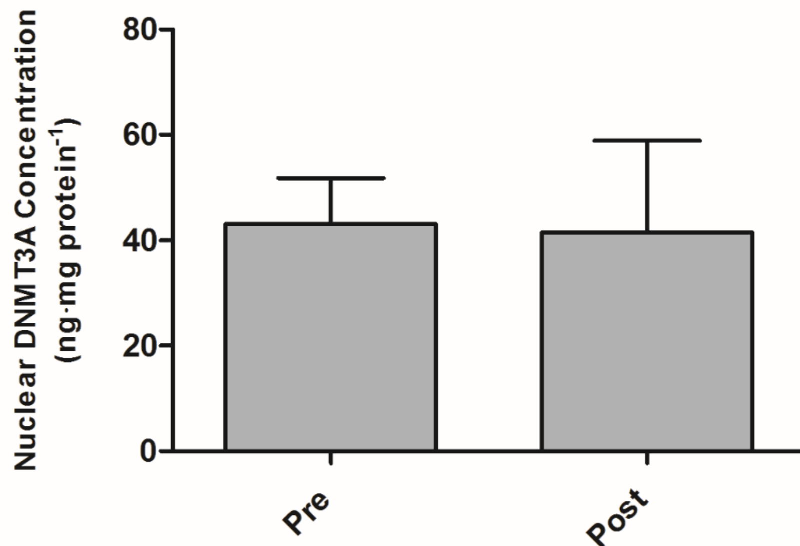

Figure 4.1 *- Mean (± SD) nuclear DNMT3A concentrations following stimulation with exercise-conditioned plasma.*

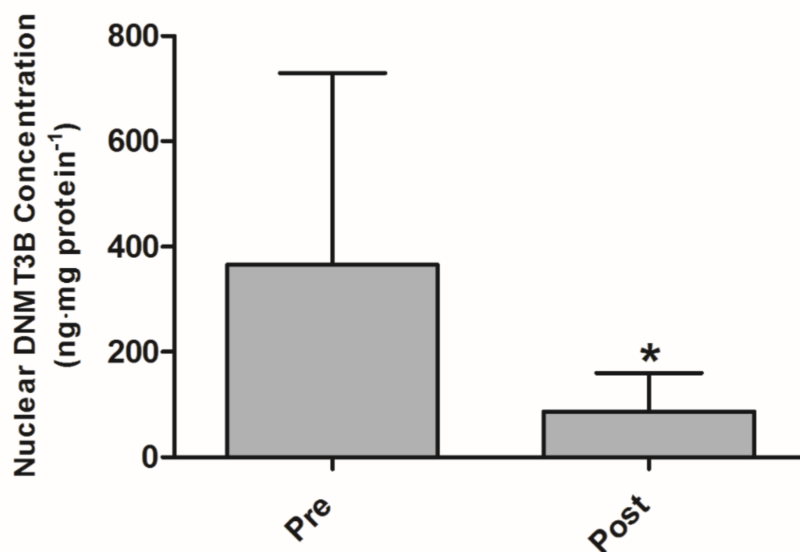

Figure 4.2 *- Mean (± SD) nuclear DNMT3B concentrations following stimulation with exercise-conditioned plasma. *significantly lower than 'pre' ($p < 0.05$).*

4.3.3 Plasma IL-6 Concentration

Plasma concentrations of IL-6 were also quantified. Figure 4.3 shows that mean (± SD) systemic concentrations significantly increased from 0.4 (± 0.3) to 14.9 (± 7.4) $pg{\cdot}ml^{-1}$; a 35-fold increase 'pre' to 'post' exercise ($p = 0.005$).

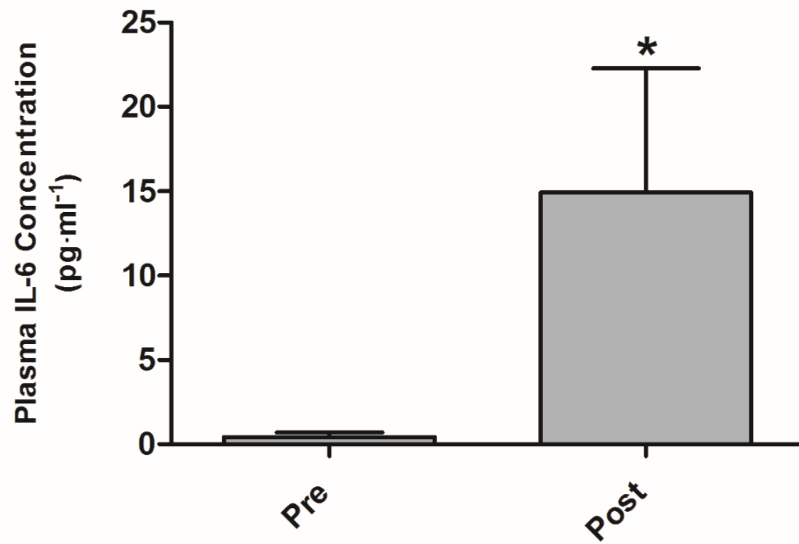

Figure 4.3 *- Mean (± SD) pre- and post-exercise plasma concentrations of IL-6. *significantly greater than 'pre' ($p < 0.05$).*

4.4 Discussion

This is the first study to investigate changes in the concentrations of the *de novo* DNA methyltransferases DNMT3A and DNMT3B following stimulation of PBMCs with plasma isolated before and after an acute bout of intense treadmill exercise. While DNMT3A concentrations remained unaltered, a significant reduction in the nuclear concentration of DNMT3B was observed which could be considered to be consistent with previous findings showing decreased *DNMT3B* mRNA in *vastus lateralis* skeletal muscle samples following exercise (Laye & Pedersen, 2010). These data suggest that the two *de novo* methyltransferases have distinct roles in response to exercise, and highlights a possible mechanism by which exercise may be able to acutely alter levels of DNA methylation.

The significant elevation in plasma IL-6 immediately following the exercise bout suggests that nuclear transport or transcription of DNMT3A is unlikely to be solely regulated by IL-6 mediated signalling pathways immediately upon cessation of exercise, in contrast to the response of DNMT3B. The posit that DNMT3A and DNMT3B possess diverse functions following an acute exercise stimulus may be supported by findings that show that the enzymes are differentially expressed during development; DNMT3B is the primary active enzyme during earlier embryonic stages such as implantation, whereas *DNMT3A* expression is greater in the later stages of embryonic development (Okano et al., 1999) and during methylation of maturing gametes (Hara et al., 2014). It is also of interest that these data corroborate the findings reported in chapter 3 that DNMT3A is less abundant within the nuclei of PBMCs, possibly suggesting that DNMT3B may be a more important regulator of DNA methylation in somatic cells.

The observed decrease in nuclear concentration of DNMT3B could be due to two mechanisms; downregulation of gene transcription and/or enhanced nuclear export. Laye and Pedersen (2010) reported a 50% decrease in skeletal muscle *DNMT3B* mRNA following three hours of cycling at 60% of v$\dot{V}O_{2max}$. Furthermore, ionomycin stimulation of differentiated myotubes, but not proliferating myoblasts, caused attenuation of both *DNMT3A* and *DNMT3B* mRNA by approximately 40%. The 75% decrease in nuclear concentration of DNMT3B within the present study suggests nuclear export of the enzymes may also be occurring concurrently with transcriptional downregulation; 60 minutes of cycling has previously been shown to significantly decrease nuclear concentrations of the class IIa histone deacetylases HDAC4 and HDAC5 in skeletal muscle, possibly due to enhanced nuclear export (McGee *et al.*, 2009) via Ca^{2+}/calmodium-dependent protein kinase (CaMK) signalling (McKinsey *et al.*, 2000). Since skeletal muscle CaMKII activity can be augmented by muscular contraction in an intensity dependent manner (Rose *et al.*, 2006), when combined with reported contraction-induced DNA hypomethylation of mouse skeletal muscle (Barrès et al., 2012; Lucas et al., 2012), this suggests a mechanistic link between exercise, specifically muscular contraction, and nuclear export of epigenetic enzymes. Furthermore, HDACs have been shown to bind DNMT1 (Fuks, Burgers,

Brehm, Hughes-Davies, & Kouzarides, 2000; Rountree et al., 2000), while DNMT3B was shown to correlate with HDAC1 and HDAC2 in ovarian cancer (Gu et al., 2013), therefore it is plausible that DNMTs are exported as part of a complex with HDACs. Whether signalling within peripheral blood cells is the same as within muscle in response to exercise remains to be elucidated.

If the observed decrease in nuclear concentration of DNMT3B did indeed translate into DNA hypomethylation, this would be in contrast to the results reported by Robson-Ansley et al. (2014), who found no differences in global or gene-specific DNA methylation following an identical protocol. However, the participants used by Robson-Ansley et al. (2014) were trained, as opposed to the participants and cell donor utilised within the present study who were not specifically endurance trained males, despite a good level of aerobic fitness. It is possible, therefore, that endurance training-associated increases in CaMKII (Rose et al., 2007) may attenuate the acute epigenetic response to a further single exercise bout. Furthermore, the Infinium HumanMethylation27 beadchip was utilised in this study which only covers 27, 578 CpG sites across 14, 495 genes, which may explain the lack of detectable changes.

The conflicting outcomes between the exercise and rIL-6 stimulation (chapter 3) data suggests that molecules found in the plasma other than IL-6 could have profound influences on nuclear concentrations of DNMTs. PGE_2, a lipid autocoid derived from arachidonic acid, is an important mediator in the acute inflammatory response, and can regulate *IL6* expression in various cell types (Bagga et al., 2003; Hinson et al., 1996; Inoue et al., 2002; Williams & Shacter, 1997). Elevated plasma PGE_2 has previously been reported following a marathon (Demers et al., 1981) and run to exhaustion at 80% of $\dot{V}O_{2max}$ (Venkatraman et al., 2001), thus, given the intensity of the exercise bout in the present study, it is possible that plasma PGE_2 was indeed elevated, contributing to the augmentation of circulating IL-6. Furthermore, expression of *Sp1* and *Sp3* transcription factors, known to regulate DNMT3A (Jinawath et al., 2005), have been shown to be upregulated following PGE_2 stimulation, concomitant with an increase in *DNMT3A* expression in fibroblasts, and a decrease in *DNMT3A* and *DNMT1* expression in RAW macrophages (Huang et al., 2012). Unfortunately, no data appears to support the supposition that DNMT3B may be altered by PGE_2, however, the available data demonstrates that PGE_2 is able to modify the expression of other epigenetic regulatory enzymes.

Circulating miRNAs are another candidate group of plasma molecules that could influence the expression or transport of DNMTs. Changes in plasma concentrations of a multitude of miRNAs have been reported following acute aerobic (Nielsen et al., 2014), resistance (Sawada, Kon, Wada, Ushida, Suzuki, & Akimoto, 2013), and eccentric (Banzet et al., 2013) exercise. The role of IL-6 in the modification of *DNMT1* expression, which has been discussed elsewhere within this paper, could be mediated by miRNA-148a and miRNA-152 (Braconi et al., 2010; Huang et al., 2010[a]). Furthermore, miRNA-143 has been shown to target DNMT3A (Ng et al., 2009), miRNA-148 targets the DNMT3B protein coding region (Duursma et al., 2008), while miRNA-29 appears to be

involved in the regulation of both DNMT3A and DNMT3B (Fabbri et al., 2007; Garzon et al., 2009; Takada et al., 2009).When combining these data, it appears entirely possible that exercise-induced changes to plasma miRNAs could have a profound influence on nuclear DNMT transport and/or gene expression.

A limitation of the present study was the lack of a direct measure of genomic or gene-specific methylation, and thus, it is not known whether the attenuation of DNMT3B resulted directly in the modification of DNA methylation. Hypomethylation is known to occur passively via downregulation of DNMT1, or actively via upregulation of TET activity (Kohli & Zhang, 2013), however, the functional effects of a reduction in DNMT3B are unknown.

It must also be noted that due to the utilisation of a standardised cell sample from a single participant in all experimental conditions, results may lack generalisability. For example, the cell donor in the present study could be a low or non-responder due to their particular genotype (Terruzzi et al., 2011) possibly accounting for the lack of change in DNMT3A concentration following stimulation with exercise-conditioned plasma.

4.4.1 Conclusion

The data presented thus far demonstrates that IL-6, recombinant or located in the plasma, exerts significant effects on *de novo* DNA methyltransferases in human PBMCs. An investigation into the distinct functions of DNMT3A and DNMT3B is necessary in order to elucidate how the enzymes are differentially regulated; whether DNMT3A exhibits a delayed response to exercise, similar to the expression of the enzyme later in embryonic development, or if it is simply unreceptive to stimulation with plasma proteins. Further studies should also look to sample at multiple timepoints following exercise bouts of varying modes and durations in order to clarifying how differential systemic changes affect nuclear localisation of these enzymes.

CHAPTER 5

THE EFFECTS OF EXERCISE-CONDITIONED PLASMA ON NUCLEAR CONCENTRATIONS OF DNMT3A, DNMT3B, AND TET FOLLOWING ULTRA-ENDURANCE EXERCISE

5 The Effects of Exercise-Conditioned Plasma on Nuclear Concentrations of DNMT3A, DNMT3B, and TET Following Ultra-Endurance Exercise

5.1 Introduction

Results presented in chapters 3 and 4 thus far strongly support the plethora of *in vitro* data that inflammation, particularly IL-6, plays a key role in the manipulation of DNMTs, and therefore, epigenetic modification. Specifically, in chapter 4, it was demonstrated that when PBMCs of a recreationally active individual were incubated in the presence of exercise-conditioned plasma, there was a significant attenuation of DNMT3B nuclear concentrations, despite no change in the lesser abundant DNMT3A. These data highlight that, similar to expression during fetal development, the two *de novo* DNA methyltransferases possess distinct roles, and also raises the possibility that *DNMT3A* transcription and/or cellular transport is either not significantly altered by changes in circulating proteins and molecules following exercise, or simply that these exercise-induced systemic changes were not large enough to elicit a response. In order to investigate this, therefore, it appeared logical to utilise an exercise bout that would elicit a much more extreme inflammatory response. Furthermore, as to whether the attenuation in DNMT3B would result in previously reported exercise-induced hypomethylation *via* passive mechanisms, or whether this would be due to TET-dependent active demethylation, remains to be clarified.

Marathon races, officially defined as a distance of 26.2 miles or 42.2 km, place an extraordinary physiological demand on the body, resulting in pronounced changes to systemic inflammatory markers. Scherr et al. (2011) reported that plasma high sensitivity C-reactive protein (hsCRP), IL-6, IL-10 and TNFα were all elevated following the Munich marathon, although no change was observed in *IL6* and *TNFα* mRNA in PBMCs (Bernecker et al., 2013). Similarly, Howatson et al. (2010) reported elevations in plasma IL-6 and hsCRP concentrations, immediately following, and up to 48 hours following the London marathon, respectively. Nieman et al. (2001) also found that circulating IL-10, IL-1ra, IL-6 and IL-8 concentrations were elevated post-marathon, in addition to small, yet significant, increases in IL-1β and TNFα. Interestingly, while previous research has identified numerous miRNAs that are transiently upregulated in plasma following exercise (Nielsen et al., 2014; Sawada et al., 2013; Banzet et al., 2013), de Gonzalo-Calvo et al. (2015) were the first to show that endurance exercise is able to elicit significant alterations to circulatory inflammatory miRNAs profiles, with a marathon causing significant augmentation of 12 of these, in contrast to a 10 km run, which only resulted in a single inflammatory miRNA to be elevated.

It is well established that ultra-endurance races, which are generally considered to consist of any distance greater than 42km covered in a standard marathon, are also able to cause considerable transient increases in plasma concentrations of a number of inflammatory markers such as hsCRP

and IL-6. Following a 50km race, plasma IL-6 was significantly elevated upon completion (Mastaloudis, Morrow, Hopkins, Devaraj, & Traber, 2004), while a 200km run, with a duration that ranged from 24 to 35 hours, caused significant augmentation of both plasma IL-6 and hsCRP, but TNFα remained unchanged (Kim et al., 2007). During races of even greater duration, such as a 48 hour ultra-marathon, IL-6 was significantly augmented by the 6 hour mark and remained elevated at 48 hours, returning to baseline another 48 hours following completion of the race (Klapcinska, Waśkiewicz, Chrapusta, Sadowska-Krępa, Czuba, & Langfort et al., 2013), while CRP was found to be elevated at 12 hours, with a further increase at 24 hours. Similar results were reported following a 308km race with a time limit of 64 hours; IL-6, IL-8, CRP, and IL-10 were all elevated by the 100km mark, and remained at a similar level for the duration of the race (Shin and Lee, 2013). More specifically to the present investigation, IL-6 was shown to be elevated immediately following a 24 hour race (Waśkiewicz et al., 2012), while more frequent measurements demonstrated that a significant increase occurred at 6 hours, with a further increase at 12 hours, which remained elevated at 24 hours (Wallberg, Mattson, Enqvist, & Ekblom, 2011). Neither study showed significant increases in TNFα or CRP immediately following the ultra-marathon, although CRP was elevated at 12 and 24 during recovery (Waśkiewicz et al., 2012).

Given the potential of singular bouts of ultra-endurance activity to cause large elevations in circulating levels of inflammatory markers such as IL-6, combined with the convincing evidence that inflammatory cytokines are able to manipulate *DNMT* expression and DNMT protein levels in a number of cell lines including PBMCs, a clear rationale underpins the use of such an intense stimulus. The primary objective of this study, therefore, was to characterise the acute response of DNMT3A and DNMT3B nuclear concentrations in PBMCs when cultured in the presence of exercise-conditioned plasma isolated from participants before and after an ultra-endurance event. Furthermore, potential changes in hydroxymethylated DNA and TET activity would provide further insight into whether exercise-induced circulatory changes affect DNA demethylation.

5.2 Methods

5.2.1 Setting and Participants

Samples were collected during the 2011 and 2012 Glenmore24 Trail Race, a 24 hour ultra-marathon over a 6km looped course, in the Cairngorms National Park situated in the Scottish Highlands. 18 (male = 15, female = 3) participants agreed to participate and gave fully informed consent. Mean (± SD) characteristics of participants at baseline are reported in table 5.1. The study received appropriate ethical approval from Coventry University Applied Research Committee and Northumbria University Ethics committee.

5.2.1.1 Sample Size Estimation

Similarly to chapter 4, power calculations were based upon initial findings presented in chapter 3 that recombinant human IL-6 was able to significantly augment nuclear concentrations of these enzymes (appendix E). However, to account for a potential high number of drop outs given the extended duration of the exercise bout, a greater number of individuals were recruited for this study. Sample size was calculated using a spreadsheet accessed from the Boston University and Boston Medical Centre Research Compliance website ("Sample-Size Calculations", 2014).

5.2.2 Data Collection

An hour prior to the start of the ultra-marathon (12:00), stature was measured using a stadiometer and body mass was measured using calibrated electronic scales (BF510, Omron Healthcare, Ukyo-ku, Kyoto, Japan). Hip and waist circumferences were measured using standard procedures outlined in chapter 2 (2.2.1). Participants then sat for a period of 10 minutes prior to blood sampling, whereby blood was collected into one 6 ml Lithium Heparin vacutainer tube (Becton Dickinson, Oxford, UK) from a vein the antecubital fossa of the forearm (section 2.2.4). Participants wore a SenseWear (7.0, Bodymedia, Pittsburgh, PA, USA) armband monitoring system to track distance and energy expenditure during the race. Immediately following the end of the race (12:00), body mass was re-measured and a blood sample was taken using the same procedure as before the race. Pre- and post-run blood samples were centrifuged at 1700*g for 10 minutes at 4 ºC in order for plasma to be separated. Samples were immediately stored at -80 ºC for later analysis. Plasma volume shift was determined as described in section 2.3.2.

5.2.3 Lab Analyses

As described in sections 2.3.4 and 2.3.5, a standardised sample of PBMCs were incubated with exercise-conditioned plasma isolated before and after exercise. Nuclear proteins were then extracted and quantified by Bradford assay prior to analysis of DNMT quantification.

5.2.3.1 Cytokine Assays

Analysis of plasma IL-1β, IL-12p70, IFN-γ, IL-6, IL-8, IL-10 and TNFα was performed using the Human ProInflammatory 7-Plex Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, USA), which detects the cytokines in a sandwich immunoassay format. Samples and solutions containing labelled detection antibodies were added to wells. Sample analytes bind to capture antibodies that are immobilised on the working electrode surface. Bound analytes then recruit labelled detection antibodies. An M1250 Sector Imager (Meso Scale Discovery, Gaithersburg, USA) was used to measure intensity of emitted light in order to quantify concentrations of the cytokines present in the samples. Lower limits of detection for each cytokine were 0.58, 0.77, 0.80, 0.18, 0.10, 0.57, and 0.28 $pg \cdot ml^{-1}$, respectively. Intra-assay coefficient of variations for each cytokine, in the order first mentioned above, were 13.88%, 9.81%, 6.31%, 9.25%, 5.37%, 7.81%, and 6.45%. Inter-assay coefficient of variations were calculated as 19.41%, 19.97%, 9.02%, 17.05%, 5.50%, 32.41%, and 21.87%.

5.2.3.2 DNMT Quantification

Quantification of nuclear DNMT3A and DNMT3B enzyme concentrations were performed as per manufacturer guidelines described in section 2.3.8 (Epiquik DNMT3A/B Assay Kit, Epigentek, New York, USA). All samples were assayed in duplicate on a single plate. Intra-assay coefficient of variations for DNMT3A and DNMT3B were calculated as 10.47% and 4.15%, respectively.

5.2.3.3 Hydroxymethylated DNA and TET Activity

Quantification of TET activity from nuclear extracts was performed as per manufacturer guidelines (Epigenase 5mC Hydroxylase TET Activity/Inhibition Assay Kit, Epigentek, New York, USA). The assay involves a methylated substrate being coated onto the microplate well, with TET activity calculated from the conversion of methylated to hydroxymethylated products. Microplates were scanned at 450 nm with a reference wavelength of 655 nm (Synergy HT, BioTek, Winooski, USA). The following equations were used to quantify amount of hydroxymethylated product and TET activity:

$$\textit{Hydroxymethylated product (ng)} = \textit{(sample OD} - \textit{blank OD)/slope}$$

$$\textit{TET activity } (ng \cdot min^{-1} \cdot mg^{-1}) = \textit{hydroxymethylated product (ng)/(protein amount } (\mu g) * min) * 1000$$

OD is optical density/absorbance; Slope is generated from the standard curve; Protein amount (μg) as quantified by Bradford assay; 'Min' is incubation time.

5.2.4 Statistical Analysis

Data was analysed using IBM SPSS Statistics version 22. Prior to performing parametric testing of the data, the Shapiro-Wilk test of normality was utilised. Non-normally distributed data was

subsequently $\log_{10}$ transformed. Paired Samples T Tests were used to test for significance of repeated measures data. A Pearson product-moment correlation was also used. Multiple regression analysis was then run to investigate whether significant changes in pro-inflammatory cytokines predicted change in DNMT nuclear concentration. Statistical significance was set as $p \leq 0.05$.

5.3 Results

5.3.1 Participant Characteristics

Mean characteristics of the participants are provided in Table 5.1. Mean body mass index shows that participants were within the normal healthy range of less than 25 kg·m^2. Waist circumference and waist to hip ratio classify the participants as healthy with low risk of disease (ACSM, 2014). Tables 5.2 shows the average distance covered, energy intake, and energy expenditure during the race. Table 5.3 provides a more detailed breakdown of macronutrient intake during the race.

Table 5.1 *- Mean (± SD) participant characteristics at baseline (n = 18).*

Variable	Mean (± SD)
Age (years)	40 (± 8)
Stature (cm)	176.8 (± 8.1)
Mass (kg)	78.9 (± 12.0)
Body Mass Index (kg·m^2)	25.1 (± 2.7)
Waist Circumference (cm)	83.4 (± 9.2)
Hip Circumference (cm)	98.3 (± 6.1)
Waist to Hip Ratio	0.85 (± 0.05)

Table 5.2 *- Mean (± SD) distance and energy expenditure, and intake (n = 18).*

Variable	Mean (± SD)
Distance Covered (km)	157.1 (± 27.0)
Energy Expenditure (kcal)	13169 (± 2815)
Energy Intake (kcal)	5062 (± 2880)

Table 5.3 *- Mean (± SD) macronutrient intake during the race (n = 16).*

Macronutrient	Mean (± SD)
Protein (g)	90.8 ± 41.7
Fat (g)	121.5 ± 54.6
Carbohydrate (g)	901.4 ± 617.3
Protein (%)	7.8 ± 2.8
Fat (%)	23.2 ± 8.7
Carbohydrate (%)	69.0 ± 10.8

5.3.2 DNMT Concentrations

The effects of cell stimulation with plasma isolated following a 24 hour ultra-endurance race on subsequent changes in nuclear concentrations of the *de novo* DNA methyltransferases DNMT3A and DNMT3B are illustrated visually in figures 5.1 and 5.2. Nuclear concentrations of DNMT3A significantly increased from 20.2 (± 36.7) to 86.6 (± 138.7) ng·mg protein^{-1} ($p = 0.031$), despite the large variation in post-exercise concentrations. There was no significant change in DNMT3B nuclear concentration however (412.2 (± 107.2) to 441.9 (± 143.9) ng·mg protein^{-1})($p = 0.514$).

Nuclear concentrations of DNMT3B were significantly greater than DNMT3A at both timepoints ($p < 0.001$).

5.3.3 TET Activity

No TET-converted hydroxymethylated product was detected for any of the experimental samples, indicating either a lack of TET activity, or that activity levels were lower than the sensitivity of the assay. Blank and standard controls verified that the assay functioned correctly.

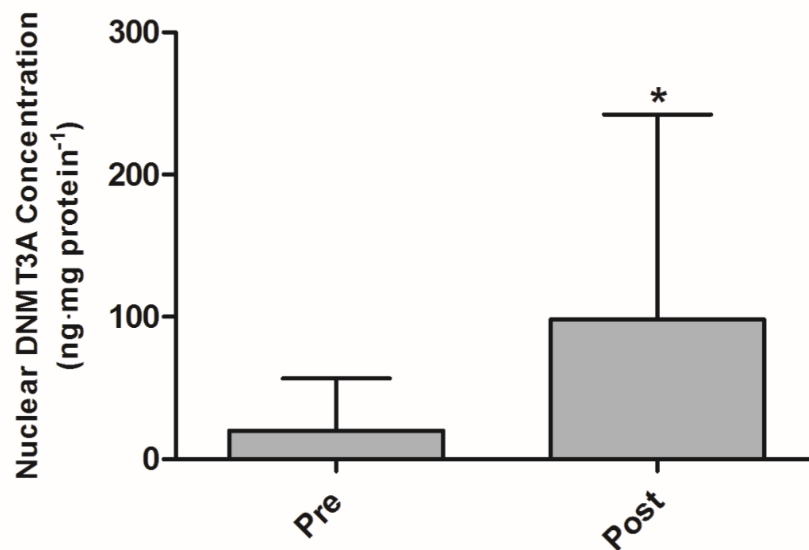

Figure 5.1 *- Mean (± SD) nuclear DNMT3A concentrations following stimulation with exercise-conditioned plasma. *significantly greater than 'pre' (p < 0.05).*

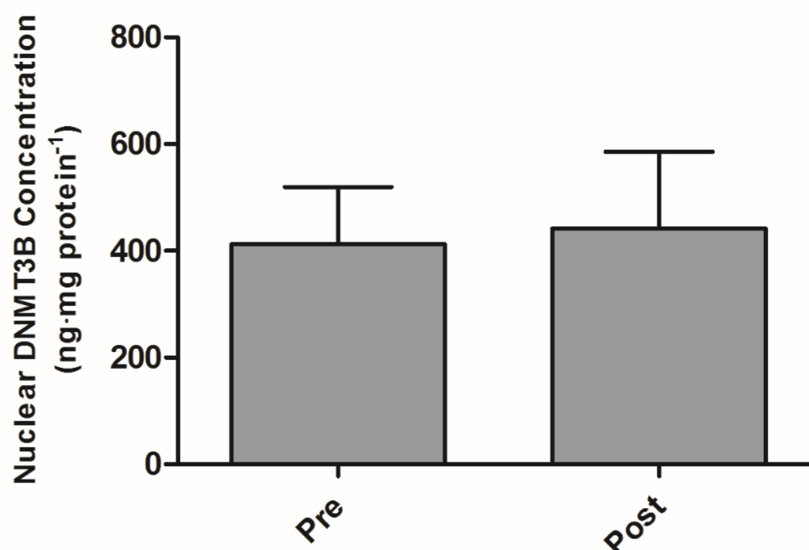

Figure 5.2 *- Mean (± SD) nuclear DNMT3B concentrations following stimulation with exercise-conditioned plasma.*

5.3.4 Plasma Pro-inflammatory Cytokines Concentrations

Significant increases in plasma IL-10 (4.36 to 11.98 pg·ml^{-1}; $p < 0.001$), IL-6 (0.41 to 13.12 pg·ml^{-1}; $p < 0.001$), IL-8 (12.79 to 34.26 pg·ml^{-1}; $p < 0.001$) and TNFα (2.85 to 3.47 pg·ml^{-1}; $p = 0.002$) were observed following the race (figures 5.3 to 5.6). No significant changes were observed in plasma concentrations of IFN-γ, IL-12 p70 or IL-1β (p = 0.532, 0.663, 0.993) (see table 5.4).

Table 5.4 *- Mean (± SD) pre- and post-exercise plasma concentrations of IFN-γ, IL-12 p70, and IL-1β (n = 18).*

Timepoint	**Mean (± SD) IFN- γ Concentration (pg·ml^{-1})**	**Mean (± SD) IL-12 p70 Concentration (pg·ml^{-1})**	**Mean (± SD) IL-1β Concentration (pg·ml^{-1})**
Pre-exercise	1.01 (± 0.78)	22.04 (± 63.54)	0.10 (± 0.29)
Post-exercise	1.11 (± 1.64)	19. 12 (± 53.78)	0.52 (± 1.01)

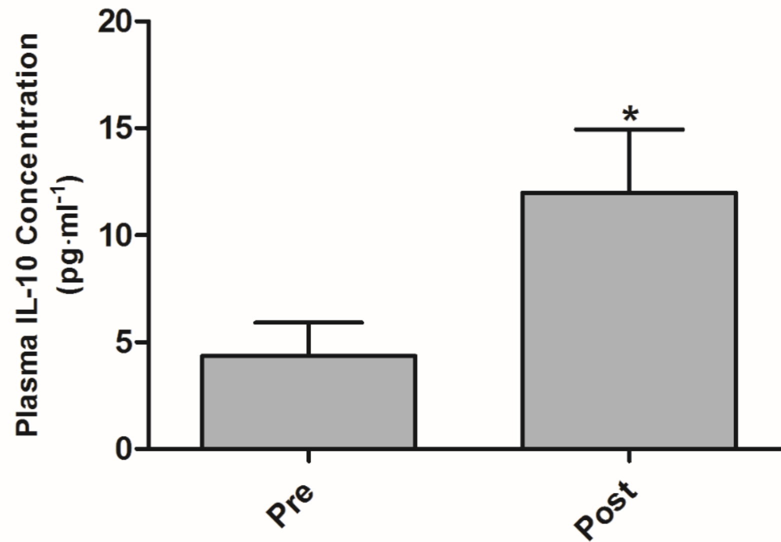

Figure 5.3 *- Mean (± SD) pre- and post-exercise plasma concentrations of IL-10. *significantly greater than 'pre' ($p < 0.05$).*

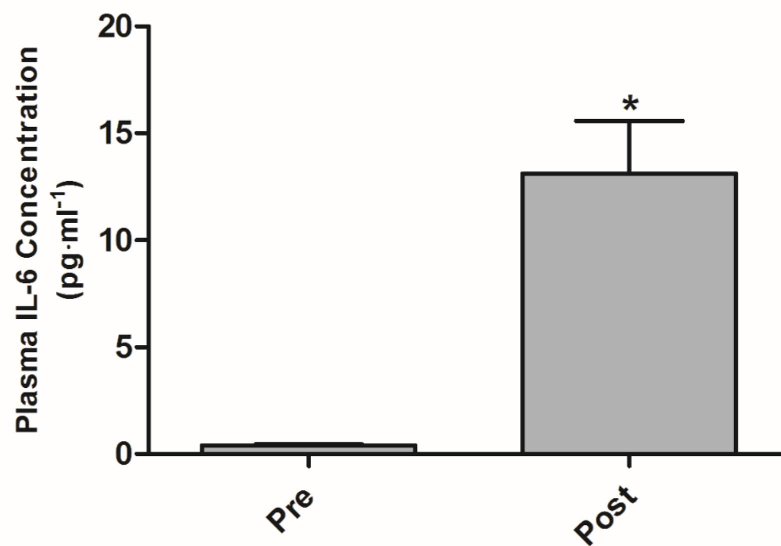

Figure 5.4 *- Mean (± SD) pre- and post-exercise plasma concentrations of IL-6. *significantly greater than 'pre' ($p < 0.05$).*

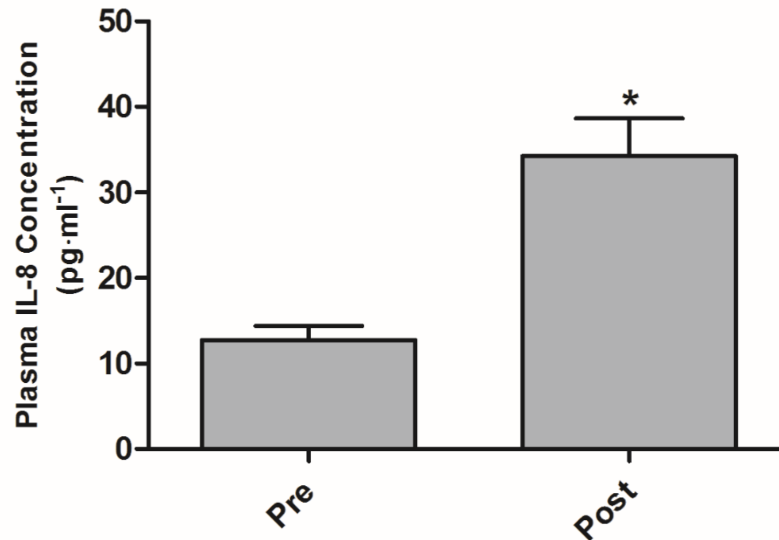

Figure 5.5 *- Mean (± SD) pre- and post-exercise plasma concentrations of IL-8. *significantly greater than 'pre' (p < 0.05).*

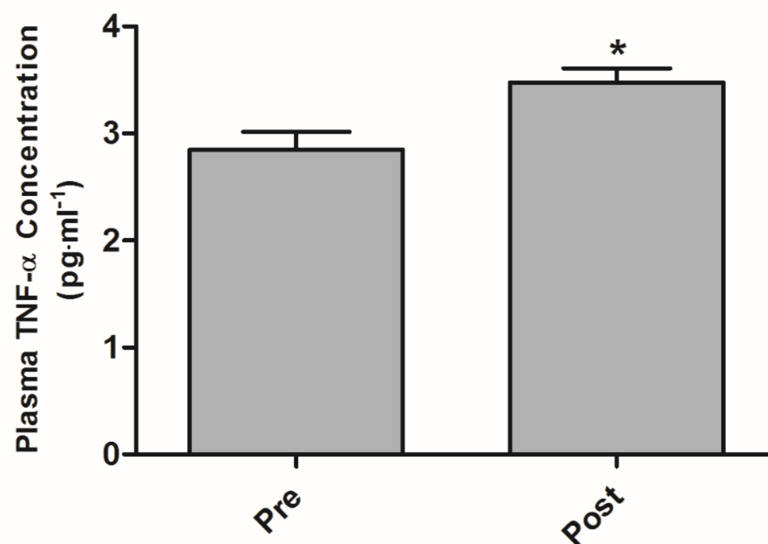

Figure 5.6 *- Mean (± SD) pre- and post-exercise plasma concentrations of TNFα. *significantly greater than 'pre' (p < 0.05).*

5.3.5 Linear and Multiple Regression

The elevation in IL-10 was significantly correlated with distance covered during the race (R = 0.691, $p = 0.002$). Similarly, the significant augmentation of plasma IL-6 was also correlated with distance covered (R = 0.632, $p = 0.005$). IL-6 and IL-10 were strongly correlated with each other (R = 0.851, $p < 0.001$). However, distance was not correlated with the change in plasma concentrations of either IL-8 or TNFα.

The augmentation of nuclear DNMT3A was not correlated with any of the significantly elevated plasma cytokines individually. Multiple regression analysis showed that significant elevations in

plasma concentrations of IL-10, IL-6, IL-8, and TNFα together were unable to predict the change in nuclear concentrations of DNMT3A (R = 0.598, $R^2 = 0.358$, $p = 0.477$).

5.4 Discussion

This is the first study to investigate epigenetic changes in response to circulatory proteins and molecules found in the plasma following an ultra-endurance event. Given the previously reported data showing that circulating inflammatory cytokines, in particular IL-6, appear to exert significant influence over nuclear localisation of the *de novo* DNMTs, it was hypothesized that ultra-endurance exercise-conditioned plasma would also elicit significant changes in nuclear concentrations of DNMT3A and DNMT3B. In contrast to our previous study whereby DNMT3B nuclear concentrations, but not DNMT3A, were attenuated following stimulation with exercise-conditioned plasma, we observed a significant augmentation of DNMT3A, with no significant change in DNMT3B. Data corroborated that DNMT3A is less abundant than DNMT3B within the nucleus at baseline. Furthermore, the amount of hydroxymethylated product was presumed to be out of the detection range of the assay used, and therefore, TET activity was unable to be measured. Manipulation of DNMT nuclear concentration following exercise has not yet been investigated following endurance exercise of extended duration, with the 120 minute protocol utilised in chapter 4 being the only known example of this type of investigation. Since identical cells were utilised in both, there were no differences in cellular adaptation to previous training or genetic susceptibility to epigenetic modification. Differences between the studies, therefore, must be due to exercise-induced circulatory changes.

Post-exercise augmentation of IL-6, IL-10, and IL-8 plasma concentrations corroborates previous research into ultra-endurance events (Kim et al., 2014; Klapcinska et al., 2013; Mastaloudis et al., 2004; Shin and Lee, 2013; Wallberg et al., 2011; Waśkiewicz et al., 2012). The response of TNFα to longer endurance events is inconsistent however; similarly to the present study, a small, but statistically significant augmentation occurred following a marathon (Nieman et al., 2001; Scherr et al., 2011) and 50km race (Mastaloudis et al., 2004). In contrast, Kim et al. (2014) and Wallberg et al. (2011) were unable to detect significant changes, although considering that all of the previously reported increases in TNFα were small, lack of significance may simply be due to differential statistical power between studies.

The systemic IL-6 response is thought to be largely dependent on duration of exercise (Fischer, 2006); depletion of muscle glycogen stores during a race of this length would be expected to cause upregulation of the *IL6* gene in order to signal the liver to enhance hepatic glucose output (Keller et al., 2001). Furthermore, since the distance covered during the race and elevation in plasma IL-6 concentration were significantly correlated, this also supports the fact that elevated plasma IL-6 is a product of extreme metabolic demand. Exercise-induced perturbations in $Ca2^{+}$ and oxidative stress are also likely to have occurred, which are known to upregulate IL-6-activating transcription factors (Fischer, 2006).

IL-10 concentrations were correlated with both distance covered and IL-6, suggesting that the elevation in this anti-inflammatory cytokine could be due to a homeostatic mechanism by which the body attempts to resolve systemic inflammation, as previously shown in participants infused with recombinant IL-6 (Steensberg, Fischer, Keller, Møller, & Pedersen, 2003).

Nieman et al. (2003) have previously reported that elevated circulating IL-8 is not due to local production, but likely due to augmented expression in PBMCs. Given the role of IL-8 in neutrophil chemotaxis and leukocyte activation, it is unsurprising that systemic concentrations were elevated following such an extreme exercise bout, in order to enhance neutrophil migration (Risoy et al., 2003) which begins resolution of muscle damage immediately upon cessation of exercise (Peake, Nosaka, & Suzuki, 2005). Conversely however, TNFα has been hypothesized to be released locally from exercising muscle, as Bernecker et al. (2013) reported no significant change in *TNFα* expression in PBMCs following a marathon. In line with previous research, it is clear that prolonged endurance exercise such as an ultra-marathon results in significant physiological demand, leading to a systemic inflammatory state.

In contrast to the previous study whereby nuclear DNMT3A concentration remained unaltered, a significant augmentation was observed in the present investigation. Post-exercise IL-6 reached similar concentrations in both studies, and therefore, the differential response of the *de novo* DNA methyltransferase is unlikely to be due this cytokine alone. While plasma concentrations of other inflammatory cytokines were not quantified in the previous study, TNFα, IL-8, and the anti-inflammatory IL-10 were significantly higher post ultra-marathon. A possible hypothesis could be that the combination of cytokines caused significant transcriptional upregulation of *DNMT3A* compared with each cytokine in isolation; Dhar et al. (2013) reported that 100 $ng \cdot ml^{-1}$ of TNFα and IGF-1 in combination caused an augmentation in *DNMT1* and *DNMT3B* mRNA compared with each cytokine alone, and *DNMT3A* compared with controls. Although this study was conducted using coronary artery cells, it highlights the possibility of differential DNMT regulation in response to combinations of cytokines. Another possible explanation for the lack of corroboration between studies is that although plasma IL-6 was augmented following the 120 minute protocol, IL-10, in its role in as an anti-inflammatory mediator, may not have been elevated at the time of the post-exercise sample. In contrast, the post-exercise timepoint in the present study was 24 hours from initiation of exercise, and thus, a 2-fold increase in IL-10 most likely indicates the beginning of inflammatory resolution. Given that different signalling cascades control pro- and anti-inflammatory pathways, at least with regard to IL-6 (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011), it is plausible that differential activation of these pathways due to the timeframe in which the samples were taken would be sufficient to elicit the contrasting downstream changes in nuclear DNMT concentrations. Multiple regression analysis demonstrated that the observed elevations in plasma concentrations of IL-6, IL-8, IL-10, and TNFα, at least alone, do not explain

the change in DNMT3A following exercise however, and thus, other exercise-induced circulatory changes must be accountable.

Oxidative stress induced by accelerated oxygen radical generation during exercise can significantly alter the systemic environment. Neutrophil activation and subsequent infiltration into skeletal muscle in response to mechanical damage and acute oxidative stress, for example due to mitochondrial reactive oxygen species (ROS) production (Ji, 1999), is one of many sources of ROS during extended endurance exercise such as a marathon (Hessel, Haberland, Müller, Lerche, & Schmike, 2000; Knez, Coombes, & Jenkins, 2006). Consequently, aerobic activity has often been shown to alter GPx activity (Hessel et al., 2000; Tauler, Aguiló, Gimeno, Guix, Tur, & Pons, 2004) and GSH redox status (Sastre et al., 1992; Viña, Sastre, Asensi, & Packer, 1995), although not definitively, as Miyazaki et al. (2001) were unable to detect any changes in GPx activity following cycling to exhaustion. Although plasma GPx is only one of many indirect markers of oxidative stress, it is of interest within the present context given the overlap between GSH and SAM synthesis. As discussed in section 1.2.5, the methyl donor SAM and antioxidant enzyme GPx both require methionine in order to be resynthesized, and thus, exercise-induced oxidative stress may disrupt the methylation cycle via this competing demand for methionine (Hitchler & Domann, 2007). Furthermore, the addition of adenosine to methionine to yield SAM is dependent on MAT activity, which itself is attenuated in an oxidised environment (Pajares et al., 1992). Wongpaiboonwattana et al. (2013) reported global (LINE-1) hypomethylation in bladder cancer cells treated with H_2O_2, which is consistent with this hypothesis. Seemingly paradoxically however, *RUNX3* was hypermethylated, similar to the results of Barrès et al. (2012), who reported augmented promoter methylation of *TFAM*, *MEF2A*, and *CS* in rat skeletal muscle myotubes following H_2O_2 treatment. It is clear that a link exists between oxidative stress, and therefore exercise-induced ROS production, and methylating capacity, although it remains to be elucidated whether DNMT or TET localisation or transcription are affected, or whether DNA methylation is influenced solely by methyl group unavailability.

Following aerobic exercise, changes to extracellular concentrations of Ca^{2+} occur in the form of elevated plasma or serum Ca^{2+} (Ljunghall et al., 1984; Salveson et al., 1994; Vora, et al., 1983). B cells sense extracellular Ca^{2+} which can activate the transcription factor NF-κB (Hammond, White, Tomic, Shi, & Spaner, 2007), while Ca^{2+} signalling in T cells promotes extracellular Ca^{2+} entry via specific channels, which also causes NF-κB activation (Feske, 2007). In turn, this can then cause downstream upregulation of *IL6* (Shimizu, Mitomo, Watanabe, Okamoto, & Yamamoto, 1990). Furthermore, human peripheral blood monocytes possess extracellular calcium receptors (Yamaguchi et al., 1998); *in vivo* and *in vitro* treatment of these cells with extracellular Ca^{2+} has been shown to directly stimulate IL-6 release (Bornefalk, Ljunghall, Lindh, Bnegston, Johansson, & Ljunggren, 1997). Our observations that rIL-6 is able to augment nuclear concentrations of DNMT3A and DNMT3B, when combined with extracellular calcium signalling, highlights a

potential pathway by which exercise-conditioned plasma may regulate nuclear concentrations of DNMTs. Currently however, no data exists to support or refute this hypothesis.

Exercise-induced modifications to circulating miRNAs could also influence the expression or transport of DNMTs. As highlighted in the previous chapter, acute aerobic (Nielsen et al., 2014), resistance (Sawada et al., 2013), and eccentric (Banzet et al., 2013) exercise elicit significant changes in plasma concentrations of a multitude of miRNAs. The role of IL-6 in the modification of *DNMT1* expression (section 1.5.1), could be mediated by miRNA-148a and miRNA-152 (Braconi et al., 2010; Huang et al., 2010[a]). Additionally, specific miRNAs display affinity for *de novo* DNA methyltransferases; miRNA-143 has been shown to target DNMT3A (Ng et al., 2009), miRNA-148 targets the DNMT3B protein coding region (Duursma et al., 2008), while miRNA-29 appears to be involved in the regulation of both DNMT3A and DNMT3B (Fabbri et al., 2007; Garzon et al., 2009; Takada et al., 2009). When combining these data, it appears entirely possible that exercise-induced changes to plasma miRNAs could have a profound influence on nuclear DNMT transport and/or gene expression. More specifically within the domain of exercise and inflammation, Gonzalo-Calvo et al. (2015) demonstrated that circulatory inflammatory miRNA concentrations were altered in a duration/distance-dependent manner, which could begin to clarify the mechanisms behind the lack of corroboration between this study and the data presented in the previous chapter.

No change in hydroxymethylated product (i.e. 5-hmC) was detected by our assay, thus we were unable to calculate TET activity based upon this. Expression of *TET2* has previously been reported in monocytes (Klug et al., 2013) and T lymphocytes (Ichiyama et al., 2015), whilst Nicoletti et al. (2016) have successfully utilised a different method (Epigentek Methylflash fluorescence) for the calculation of hydroxymethylation in PBMCs, therefore, the results of the present investigation may be due to the utilisation of an ineffective method for quantifying 5-hmC and TET activity, as opposed to a lack of transcriptional activation. More sensitive techniques for the detection of TET must be applied before any assertions can be made regarding the role of these demethylation enzymes in exercise-induced epigenetic modification.

Utilising our previously reported method of isolating cells from a resting participant, we have been able to control for polymorphisms which may affect susceptibility to epigenetic modification (Terruzzi et al., 2011) following exercise, in addition to exercise-induced changes to the cellular composition of PBMCs which would likely to change given the intensity of the exercise bout (Gonzalo-Calvo et al., 2015). As such, the methods have allowed us to isolate the effects of circulatory proteins on DNMT and TET concentrations in PBMCs, which are likely to be the mediators of exercise-induced epigenetic modifications.

An ultra-endurance marathon is an extreme example of acute exercise, and thus, generalisability of the data to more common durations and intensities of exercise is difficult. In addition, although the use of a single set of cells controls for polymorphisms which may alter susceptibility to epigenetic

modification, it is unclear as to whether the results would be drastically different if utilising cells from a different individual. Finally, no measure of DNA methylation itself was included in the analyses, and therefore, it is not yet known as to whether changes in DNMT3A nuclear concentration of translate to functional alterations in DNA methylation itself.

5.4.1 Conclusion

This study, when combined with the data presented thus far, further supports the role of systemic changes in inflammatory markers on exercise-induced epigenetic modifications, specifically, the *de novo* DNA methyltransferases. There has been some inconsistency between the data presented in chapters 3, 4, and 5, which is likely explained by differing circulating concentrations of inflammatory cytokines and/or miRNAs associated with different durations or intensities of aerobic exercise.

Whether the observed alterations in nuclear concentrations of these *de novo* DNA methyltransferases are due to cellular translocation or transcriptional changes has not yet been elucidated. Also of interest is whether the two enzymes exhibit a differential time course of change following exercise and into the recovery period. Furthermore, given that other types of exercise generally exhibit vastly differing systemic responses to endurance running, it seems logical to investigate a more diverse range of modalities.

CHAPTER 6

THE EFFECTS OF EXERCISE-CONDITIONED PLASMA ON NUCLEAR AND CYTOPLASMIC CONCENTRATIONS OF DNMT3A, DNMT3B, AND GLOBAL DNA METHYLATION FOLLOWING DAMAGING ECCENTRIC EXERCISE

6 The Effects of Exercise-Conditioned Plasma on Nuclear and Cytoplasmic Concentrations of DNMT3A, DNMT3B, and Global DNA Methylation Following Damaging Eccentric Exercise

6.1 Introduction

In the previous chapters, in line with a plethora of *in vitro* data, results showed that exercise-induced elevations in plasma concentrations of inflammatory cytokines, in particular IL-6, appear to regulate nuclear concentrations of DNMT3A and DNMT3B. However, the differential results between the exercise bouts utilised in chapters 4 and 5 highlights the distinct roles of the two *de novo* methyltransferases in response to exercise, and raises the question as to whether DNMT3A exhibits a delayed response to exercise-induced circulatory changes. Furthermore, discordance between rIL-6 and exercise-conditioned plasma conditions possibly suggests that other proteins or molecules located in the plasma exert a significant regulatory effect on DNA methylation in addition to inflammatory cytokines.

To the author's knowledge, there have been no investigations into the effects of other forms of acute exercise other than aerobic on DNA methylation thus far. Physiologically speaking, the demands of eccentric and plyometric exercise starkly contrasts with longer duration aerobic exercise. While prolonged endurance exercise is often associated with significant augmentation of circulating inflammatory cytokines, including IL-6 due to perturbations in calcium homeostasis, reactive oxygen species or reduced glucose availability (Fischer, 2006), short duration eccentric exercise with very little metabolic stress typically exhibits a dissimilar systemic response (Peake et al., 2005). Although changes to circulating white blood cell counts do occur, particularly following damaging exercise, it is uncommon for plasma concentrations of pro-inflammatory cytokines to be significantly elevated (Robson-Ansley, Cockburn, Walshe, Stevenson, & Nimmo, 2010) due to local anti-inflammatory regulation within the muscle itself (Peake et al., 2005). One study (Chatzinikolaou et al., 2010) did report a statistically significant elevation in plasma IL-6 concentrations following 50 hurdle jumps and 50 box jumps, however, the mean value was only approximately 3 pg·ml^{-1}, in contrast to 14.9 and 13.1 pg·ml^{-1} following the protocols in chapters 3 and 4, respectively. Furthermore, the lowest concentration of rIL-6 utilised in the pilot investigations was 10 pg·ml^{-1}, thus, despite statistical significance, a post-exercise concentration of significantly lower than 10 pg·ml^{-1} would not be considered to be clinically significant within this context.

Another possible explanation for the lack of corroboration between studies thus far is the different timecourse in which blood samples were taken; as discussed in the previous chapter, following the 120 minute protocol, IL-10 may not have been elevated at the time of the post-exercise sample, in contrast to the results of the 24 hour ultra-marathon. It is plausible, therefore, that the stark contrast

in DNMT responses between the two studies may be a product of differences in timing, as opposed to the underlying physiological demand itself. Within the context of this investigation, elevations in the plasma concentrations of IL-6 and IL-10 are likely to be small, however, other circulatory changes will occur, thus, blood samples taken over multiple timepoints throughout the study will aid in the elucidation of whether the two DNMTs differentially respond to exercise-conditioned plasma.

The aim of the present study, therefore, was to elucidate the response of nuclear concentrations of DNMT3A and DNMT3B to exercise-conditioned plasma following a bout of eccentric exercise that would likely not elicit a large, transient increase in systemic inflammatory cytokines, despite a potential local inflammatory response. Furthermore, given that the previously observed changes in nuclear concentrations could be due to two mechanisms, namely, cellular translocation or transcriptional regulation, it was decided to also quantify cytoplasmic concentrations of DNMT3A and DNMT3B as a marker of enzyme transport. This study also aimed to investigate whether modifications to nuclear concentrations of DNMTs translate to changes in global DNA methylation. Finally, the inclusion of further sample points over the three days post-exercise allowed characterisation of potential differential responses of the individual DNMTs to exercise-conditioned plasma.

6.2 Methods

6.2.1 Participants

10 recreationally active males were recruited from Northumbria University. 'Recreationally active' was defined as regularly meeting the government's exercise guidelines of 150 minutes of moderate intensity activity per week, but not currently undertaking vigorous resistance or plyometric training more than once per week. Mean (± SD) characteristics are reported in table 6.1. Participants gave fully informed written consent following the approval of all methods by the Northumbria University Ethics Committee. All PBMCs were isolated from a resting, recreationally active male who was recruited under the same criteria as the other participants, but did not take part in the remainder of the study. His characteristics fit within the standard deviation of the exercising participants' age, height, weight and BMI.

6.2.1.1 Sample Size Estimation

The sample size in the present study mimicked that of the study presented in chapter 4, which in itself was based upon pilot investigations (chapter 3) that showed significant changes in nuclear concentrations of the DNMTs following stimulation with rIL-6 (see appendix E). Sample size was calculated using a spreadsheet accessed from the Boston University and Boston Medical Centre Research Compliance website ("Sample-Size Calculations", 2014).

6.2.2 Study Restrictions

Participants were asked to abstain from any form of damaging exercise, such as resistance or plyometric training, or consuming any nutritional supplements or non-steroidal anti-inflammatory drugs (NSAIDs) for the seven days prior to the start of the study. They were asked to refrain from participating in any exercise, or consuming caffeine or alcohol for the two days immediately prior to the start of the study, until after the final measurements were taken. Participants were also required to fast for two hours immediately prior to the start of the trial, and were instructed to replicate their breakfast the morning of each day. Participants were instructed to not change their usual dietary habits during the study period, as to not cause alterations to intake of methionine, betaine, choline, and folate, which could have effects on DNA methylation.

6.2.3 Experimental Protocol

6.2.3.1 Familiarisation

A familiarisation session was conducted at least 72 hours prior to the main trial. All experimental procedures were explained, and maximal voluntary contraction (MVC) and drop jump movements

(3 maximum) were practiced by the participants with coaching on correct technique where necessary.

6.2.3.2 Main Trials

All participants began the trial between 10am and 12pm. Immediately upon arrival on the first day, stature and mass were measured using standard procedures (section 2.2.1). Participants were asked to rate muscle soreness on a 200mm visual analogue scale ranging from 'no pain' to 'extremely painful' whilst performing a squat movement with a 90° knee angle. A 10 ml venous blood sample was then taken into vacutainers containing Lithium Heparin (section 2.2.4) from a vein in the antecubital fossa region of the forearm using standard procedures. The participants then completed a five minute warm up on a motorised treadmill (Pulsar, h/p/cosmos, Germany) at a self-selected low intensity of no more than 7 km·hour^{-1}. Participants were then asked to be seated whilst a strap, attached to a strain gauge, was positioned on the ankle of their dominant leg, above the malleoli. Using a goniometer, the straps were adjusted to ensure that the knee angle was exactly 90°. 3 maximal contractions were performed with 60 seconds recovery, with the knee angle rechecked following each contraction. Following 3 MVC manoeuvres the participants completed 5 sets of 20 drop jumps from a box measuring 0.6 m. 10 seconds recovery was given between jumps, and two minutes between sets. This protocol has previously been utilised to elicit muscle damage (Goodall & Howatson, 2008; Howatson, Goodall, & van Someron, 2009; Howatson, Hoad, Goodall, Tallent, Bell, & French, 2012; Kirby, Triplett, Haines, Skinner, Fairbrother, & McBride, 2012; Miyama & Nosaka, 2004) and given that Peake et al. (2005) concluded that high intensity eccentric exercise such as downhill running or eccentric cycling results in the greatest systemic inflammatory response, this protocol was deemed appropriate for this particular investigation as it would be unlikely to elicit a large elevation in plasma inflammatory cytokines. All baseline measures were then retaken as before.

Participants then returned to the lab 2, 24, 48 and 72 hours following the completion of the drop jump protocol, and again repeated all baseline measures.

All blood samples were immediately centrifuged at 1700*g for 10 minutes at 4 °C in order for plasma to be separated and aspirated. Samples were immediately stored at -80 °C for later analysis.

6.2.4 Lab Analyses

As described full in sections 2.3.4 and 2.3.5, a standardised sample of PBMCs were incubated with exercise-conditioned plasma isolated before and after exercise. Nuclear and cytoplasmic proteins were then extracted and quantified by Bradford assay prior to analysis of DNMT quantification.

6.2.4.1 DNMT Quantification

Quantification of nuclear DNMT3A and DNMT3B enzyme concentrations were performed as per manufacturer guidelines described in section 2.3.8 (Epiquik DNMT3A/B Assay Kit, Epigentek, New York, USA). All samples were assayed in duplicate. Intra-assay coefficient of variations for DNMT3A and DNMT3B were calculated as 9.8% and 8.4%, respectively.

6.2.4.2 DNA Extraction

Separately, cell samples were diluted in order to yield $2*10^6$ cells·ml^{-1}. 250 µl of PBMCs ($0.5*10^6$ cells) were treated with 100 µl of plasma separated from whole blood of the exercising participants, and 150 µl of RPMI-1640 medium and Penicillin/Streptomycin/Glutamine solution (Sigma Aldrich, Missouri, USA), followed by incubation at 37 °C for four hours. Genomic DNA extraction was then performed as per manufacturer (Genomic DNA Extraction Kit, Abcam, Cambridge, UK) guidelines.

6.2.4.3 DNA Quantification

In order to accurately calculate the amount of methylated DNA in the next step, total amount of DNA contained in elution buffer was quantified using a spectrophotometer (Synergy HT, BioTek, Winooski, USA). UV-transparent microplates (Corning, New York, USA) containing 1 µl of DNA extract in elution buffer and 99 µl of deionised water were scanned at 260nm and 280nm, with automatic pathlength correction calculated by Gen5 software.

6.2.4.4 Global Methylated DNA Quantification

Global DNA methylation was analysed in a microplate format (Methylated DNA Quantification Kit, Abcam, Cambridge, UK), whereby DNA was bound to strip wells, and methylated DNA was detected using capture and detection antibodies which are optimised for 5-mC, with little or no cross-reactivity to hydroxymethylcytosine. All samples were assayed in duplicate, and various dilutions of the positive control (methylated polynucleotide containing 50% of 5-mC) were used to plot a standard curve. Microplates were read colorimetrically at 450nm. Intra-assay coefficient of variation was calculated as 9.7%. Lower limit of detection was 0.2 ng of methylated DNA. The following equation was used to quantify total amount of methylated DNA:

$$5\text{-}mC\ (ng) = (sample\ OD^{a} - negative\ control\ OD)/(slope * 2^{b})$$

[a] OD is optical density/absorbance;[b] Factor to normalize 5-mC in the positive control to 100%

The following equation was to use to calculate percentage of methylated DNA:

$$5\text{-}mC\ (\%) = (5\text{-}mC\ amount/input\ DNA)*100$$

6.2.4.5 Plasma Interleukin-6 Assay

As outlined in section 2.3.9, a QuantiGlo Human IL-6 ELISA (R&D Systems, Minneapolis, USA) was used in order to quantify plasma concentrations of IL-6 as per standard manufacturer guidelines. Intra-assay coefficient of variation was calculated as 5.9%.

6.2.4.6 Plasma Creatine Kinase

Plasma concentrations of creatine kinase (CK) were quantified spectrophotometrically using an automated system (Roche Modular P, Roche Diagnostics, West Sussex, UK). Lower limit of detection was 7 $U \cdot L^{-1}$, and coefficient of variation for the system was 1.93%.

6.2.5 Statistical Analysis

Data was analysed using IBM SPSS Statistics version 22. Prior to performing parametric testing of the data, the Shapiro-Wilk test of normality was utilised. Non-normally distributed data was subsequently $\log_{10}$ transformed. Following Mauchly's test of sphericity, one-way repeated measures ANOVAs with post-hoc Bonferonni adjustment for multiple comparisons were used to analyse differences in all variables between timepoints. Differences between nuclear and cytoplasmic DNMT concentrations at each timepoint were analysed using independent samples T-tests. A Pearson product-moment correlation was also run to determine the relationship between plasma IL-6 and epigenetic measures.

6.3 Results

6.3.1 Participant Characteristics

Mean characteristics of the participants at baseline are provided in table 6.1. Mean body mass index shows that participants were within the normal healthy range of less than 25 kg·m^2.

Table 6.1 *- Mean (± SD) participant characteristics at baseline (n = 10).*

Characteristic	**Mean (± SD)**
Age (years)	24 (± 5)
Height (cm)	181.1 (± 5.6)
Weight (kg)	81.6 (± 11.1)
Body Mass Index (kg·m^2)	24.9 (± 3.4)

6.3.2 Delayed Onset Muscle Soreness (DOMS)

Subjective muscle soreness was significantly elevated at all timepoints when compared with baseline ($p < 0.001$ for all comparisons), and peaked at 24h post-exercise. Although still raised at 72h post-exercise, soreness had begun to decline to pre-exercise levels. No statistical difference was observed between 24h and 48h post-exercise (figure 6.1).

6.3.3 Maximal Voluntary Contraction

Maximal force produced by the quadriceps was significantly attenuated immediately post-exercise ($p = 0.02$) and at 2h post-exercise ($p = 0.011$), which returned to pre-exercise levels by 72h (figure 6.2).

6.3.4 Plasma Creatine Kinase Activity

Significant augmentation of plasma CK occurred 2h ($p = 0.036$) and 24h post-exercise ($p = 0.035$), returning to pre-exercise activity levels by 72h post-exercise (figure 6.3).

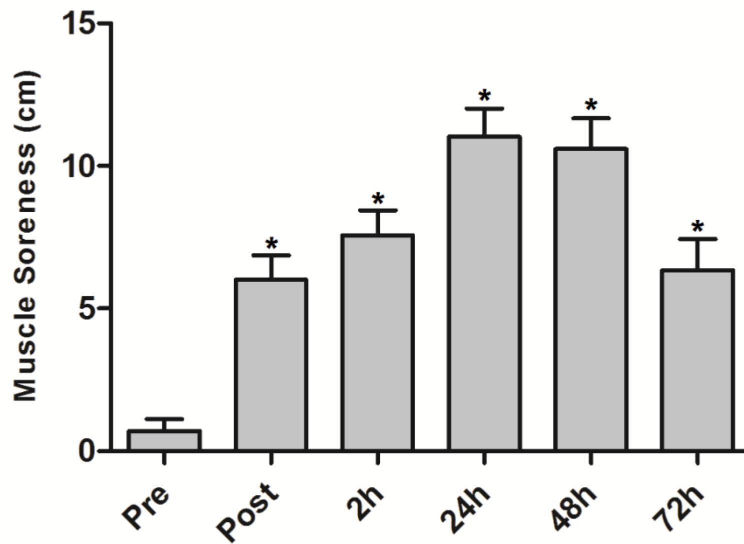

Figure 6.1 *- Mean (± SD) subjective rating of muscle soreness on a visual analogue scale. *significantly greater than 'pre' (p < 0.05).*

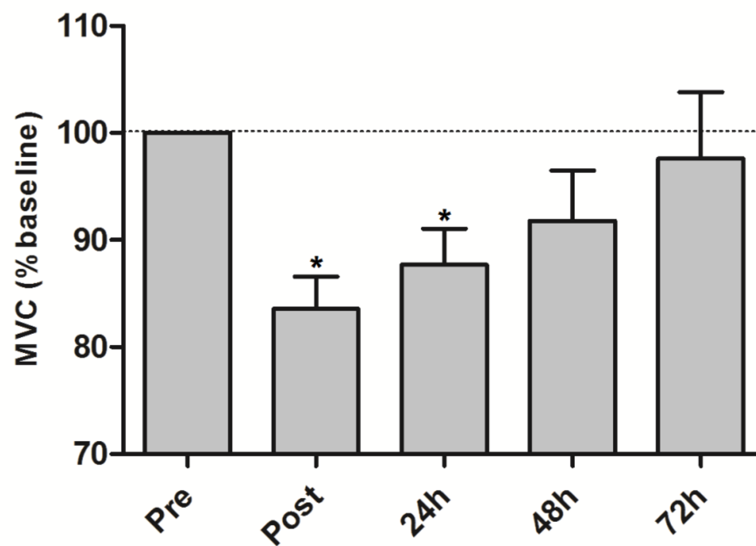

Figure 6.2 *- Mean (± SD) decrement from baseline in maximal voluntary contraction. *significantly greater than 'pre' (p < 0.05).*

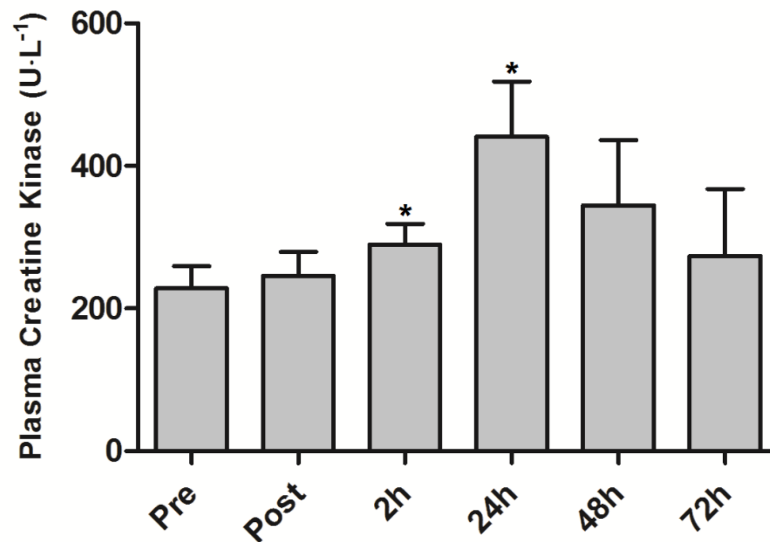

Figure 6.3 *- Mean (± SD) plasma creatine kinase activity (n = 9).*

**significantly greater than 'pre' (p < 0.05).*

6.3.5 Plasma IL-6 Concentration

A small, albeit statistically significant, elevation in plasma IL-6 concentrations occurred immediately post-exercise, returning to below baseline concentrations by 24h post-exercise (figure 6.4).

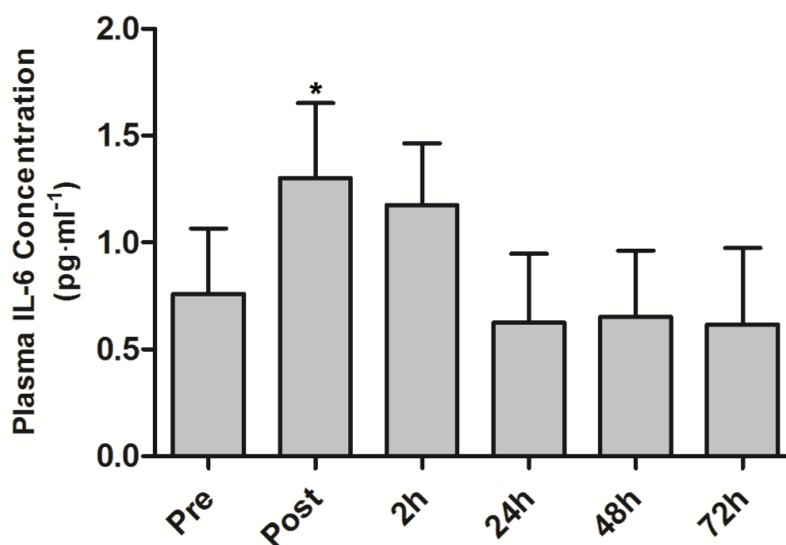

Figure 6.4 *- Mean (± SD) plasma interleukin 6 concentration.*

**significantly greater than 'pre' (p < 0.05).*

6.3.6 DNA Methyltransferase 3A Concentrations

No statistically significant changes were observed in nuclear or cytoplasmic DNMT3A concentrations over time.

Cytoplasmic concentrations of DNMT3A were significantly greater than nuclear concentrations at all time points (p = 0.018, 0.028, 0.02, 0.021, 0.001, 0.03).

6.3.7 DNA Methyltransferase 3B Concentrations

Similarly to DNMT3A, no statistically significant changes in nuclear or cytoplasmic concentrations of DNMT3B occurred at any timepoint.

Cytoplasmic concentrations of DNMT3B were significantly greater than nuclear concentrations immediately post-exercise and 24h post-exercise (p = 0.01 and 0.02, respectively).

Nuclear concentrations of DNMT3B were significantly higher than nuclear concentrations of DNMT3A at pre, post, 24h, 48h (p = 0.004, 0.002, 0.039, 0.035, respectively), while cytoplasmic DNMT3B concentrations were greater than DNMT3A at every timepoint (p = 0.005, 0.008, 0.001, 0.01, 0.001, 0.001, respectively).

Table 6.2 *- Nuclear and cytoplasmic concentrations of DNMT3A and DNMT3B at each timepoint.*

	Mean (± SD) DNMT3A Concentration (ng·mg protein^{-1})		**Mean (± SD) DNMT3B Concentration (ng·mg protein^{-1})**	
Timepoint	**Nuclear**	**Cytoplasmic**	**Nuclear**	**Cytoplasmic**
Pre	24.94 (± 17.93)	148.97 (±101.67)$^{\alpha}$	270.57 (± 200.53)$^{\beta}$	485.51 (± 235.85)$^{\gamma}$
Post	22.72 (± 16.64)	148.12 (± 114.91)$^{\alpha}$	188.03 (± 121.74)$^{\beta}$	402.22 (± 177.15)$^{\alpha\gamma}$
2h	15.93 (± 6.57)	151.61 (± 69.52)$^{\alpha}$	231.23 (± 305.31)	438.48 (± 160.24)$^{\gamma}$
24h	21.80 (± 7.14)	208.15 (± 158.27)$^{\alpha}$	213.52 (± 172.73)$^{\beta}$	512.63 (± 208.71)$^{\alpha\gamma}$
48h	18.54 (± 8.27)	182.81 (±78.94)$^{\alpha}$	225.68 (± 245.86)$^{\beta}$	416.94 (± 112.79)$^{\gamma}$
72h	27.93 (± 16.62)	171.19 (± 78.20)$^{\alpha}$	278.18 (± 244.78)	418.93 (± 131.59)$^{\gamma}$

$^{\alpha}$ significantly higher than nuclear concentrations at the same timepoint ($p < 0.05$); $^{\beta}$ significantly greater than DNMT3A nuclear concentrations ($p < 0.05$); $^{\gamma}$ significantly greater than DNMT3A cytoplasmic concentrations ($p < 0.05$)

6.3.8 Global DNA Methylation

No statistically significant modifications to the amount of global DNA methylation were observed at any timepoint (table 6.3). When corrected as a percentage of the total amount of DNA used in the assay, all timepoints remained non-significant (table 6.4).

Table 6.3 *- Mean (± SD) Global DNA methylation (ng) at each timepoint.*

Timepoint	Mean (± SD) Global DNA Methylation (ng)
Pre	0.27 (± 0.21)
Post	0.45 (± 0.46)
2h	0.31 (± 0.21)
24h	0.28 (± 0.18)
48h	0.26 (± 0.20)
72h	0.29 (± 0.27)

Table 6.4 *- Mean (± SD) Global DNA methylation (%) at each timepoint.*

Timepoint	Mean (± SD) Global DNA Methylation (%)
Pre	7.47 (± 9.95)
Post	12.70 (± 8.92)
2h	11.18 (± 13.89)
24h	10.80 (± 7.74)
48h	9.47 (± 7.50)
72h	15.44 (± 16.35)

Significant inverse relationships between plasma IL-6 and global methylation occurred at pre-exercise ($r = -0.687$, $p = 0.041$) and 48h post-exercise ($r = -0.696$, $p = 0.037$).

6.4 Discussion

All measures of muscle damage corroborate previous studies that also utilised the same drop jump protocol (Goodall & Howatson, 2008; Howatson et al., 2009; Howatson et al., 2012; Kirby et al., 2012; Miyama & Nosaka, 2004). Significant elevations in muscle soreness and plasma CK activity were observed, both peaking at 24h post-exercise, whilst a large decrement in MVC score occurred immediately following the drop jumps. Despite the statistically significant post-exercise elevation in plasma IL-6, this would still be considered to be a relatively low concentration in comparison to the concentrations of rIL-6 utilised to treat PBMCs in chapter 3, or compared to the post-exercise concentrations observed in chapters 4 and 5. As such, the lack of significant changes in any of the epigenetic measures in the present investigation may suggest, as previously hypothesized, that large, transient exercise-induced increases in plasma inflammatory markers, such as IL-6, regulate cellular translocation and/or transcriptional regulation of DNMT3A and DNMT3B. Conversely, it appears that circulatory changes following an acute bout of damaging exercise exert very little influence over nuclear or cytoplasmic DNMT concentrations or on the methylation of DNA at a global level.

Despite there not being any statistically significant alteration in nuclear or cytoplasmic concentrations of DNMT3B at any timepoint, the fact that cytoplasmic concentrations are significantly greater than nuclear concentrations immediately post- and 24h post-exercise may aid in the explanation of the previously observed post-exercise attenuation of nuclear DNMT3B protein concentration, hypothesized to be due to cytoplasmic translocation of the enzyme. As to whether transcriptional changes of the DNMT3B gene occur following exercise remains to be elucidated however. Furthermore, greater nuclear concentrations of DNMT3B, compared with DNMT3A at the majority of timepoints including at baseline, corroborates previous data (chapters 4 and 5) that shows that DNMT3B appears to more abundant than DNMT3A within the nucleus.

Given that no significant changes occurred to nuclear concentrations of either *de novo* DNMT, it is unsurprising that there was no observable change in the amount of globally methylated DNA, which appears to corroborate Robson-Ansley et al's (2014) previous findings, albeit whilst utilising an exercise bout that exerts a distinct physiological demand. Potential gene-specific methylation alterations must also not be ruled out. Interestingly, strong inverse relationships between plasma IL-6 concentrations and the amount of globally methylated DNA were discovered at the pre-exercise and 48h post-exercise timepoints. While this finding is difficult to interpret within the context of the other data, it could suggest that small fluctuations in plasma IL-6 concentrations may contribute to global hypomethylation, or at least, there is a shared underlying cause. Disruptions in Ca^{2+} homeostasis could be one such shared cause given that caffeine, which raises intracellular Ca^{2+}, can cause promoter hypomethylation in skeletal muscle Barrès et al. (2012), while ionomycin has been shown to upregulate *IL6* in L6 myotubes *in vitro* (Chan, McGee, Watt, Hargreaves, & Febbraio, 2004[a]).

Although much ambiguity remains over the effect of circulatory changes following exercise on regulatory epigenetic enzymes, one thing the data clearly shows is that aerobic and eccentric exercise differentially affect nuclear concentrations of the *de novo* DNMTs. It is well known that exercise results in acute perturbations in Ca^{2+} homeostasis, with damaging eccentric exercise generally causing an increased influx of Ca^{2+} into the cell via stretch-activated channels and/or disrupted cell membranes (Hyldahl and Hubal, 2014), whereas following aerobic exercise, changes to extracellular concentrations of Ca^{2+} occur in the form of increased plasma or serum Ca^{2+} (Ljunghall et al., 1984; Salveson et al., 1994; Vora et al., 1983). The method utilised within the present investigation was able to control for cellular perturbations in Ca^{2+} by using a standardised, 'resting' cell sample in all conditions, however, exercise-conditioned plasma isolated following aerobic exercise as in the case of chapters 4 and 5 may have altered nuclear concentrations of DNMTs via the pathways discussed in section 5.4, thus helping to explain the differential response between the types of exercise.

Again, as discussed in section 5.4, plasma miRNA concentrations have been shown to be differentially regulated by bouts of eccentric (Banzet et al., 2013), resistance (Sawada et al., 2013), and aerobic exercise (Nielsen et al., 2014). Adaptation to exercise is thought to occur as a result of repeated exposure to a stimulus, resulting in a steady-state level of a protein with a beneficial function. Aerobic and resistance or eccentric exercise elicit vastly different adaptations (Coffey & Hawley, 2007), and given that changes in gene transcription that cause more favourable phenotypes is an integral part of adaptation, it seems logical that differential epigenetic regulation would also occur following varying modes of exercise. Furthermore, it has been shown that contrasting plasma miRNA profiles are exhibited by endurance or strength trained athletes, indicating a further divide in the epigenetic response of different exercise modalities (Wardle et al., 2015). It is plausible, therefore, that exercise-induced changes to plasma miRNAs could be involved in exercise-induced modifications to DNMTs and DNA methylation, and could be a factor that helps to explain the varying responses of PBMCs stimulated with plasma isolated following different types of exercise.

To the authors' knowledge, no published data has yet reported links between epigenetic modifications and any form of exercise other than aerobic, thus, it is possible that the enzymes or cells that were investigated within this study are simply not responsive to systemic changes associated with eccentric exercise. For example, eccentric exercise may be specifically associated with gene-specific hypomethylation, in which case, the TET family of proteins, regulators of active demethylation, may be more sensitive markers. In addition, the cells that comprise the PBMC population play essential roles within the immune system, and thus, an exercise bout that elicits significant changes in systemic inflammation might also be expected to cause genetic or epigenetic changes, and vice versa, a lack of circulating inflammatory proteins may result in little to no change in these cells. These mechanisms are merely speculative due to the current lack of published findings within this field.

A limitation of the global methylation assay utilised within the present investigation was that it utilised a monoclonal anti-5mC antibody which recognises any methylcytosine, including 5-mC and 5-methyldeoxycytidine in both CpG and non-CpG sites, and therefore, lacks specificity toward 5-mC i.e. methylation at CpG sites only. The assay did, however, distinguish between 5-mC and 5-hmC, which is involved in the TET-mediated active demethylation process.

Another potential limitation is that we did not include any measures of oxidative stress. There is a close link between GPx and DNA methylation; glutathione biosynthesis requires homocysteine, which itself is synthesised from methionine, the methyl group DNMTs transfer from SAM to cytosine to create 5-mC (Hitchler & Domann, 2007). This process seems unlikely to affect transcription or cellular transport of DNMT3A and DNMT3B, but unavailability of methionine due to an exercise-induced demand for GPx could have a subsequent knock on effect on methylation of DNA.

Although participants were instructed to consume the same breakfast on all testing days, this was not provided, and therefore, not controlled for between participants. However, there was unlikely to be any acute changes in methionine, betaine, choline, or folate availability due to the instructed consumption of their regular diet on all days, and therefore, SAM availability as a methyl donor was also not likely to have been altered.

The data presented in chapters 4 to 6 shows that considerable inter-individual variation exists in concentrations of the *de novo* DNA methyltransferases as quantified by ELISA-based methods. For example, within this chapter, despite visually much greater nuclear concentrations of DNMT3B compared with DNMT3A 2h and 72h post-exercise, the extremely large variation in DNMT3B concentrations has likely contributed to the lack of a statistically significant finding. Future research should look to incorporate other methods such as qPCR to provide a more definitive quantitative measure of changes in these enzymes.

6.4.1 Conclusion

This is the first study to investigate exercise-induced modifications to DNA methylation and the regulatory enzymes following an intense bout of damaging eccentric exercise, and suggests that small increases in systemic inflammation are likely to be insufficient to drastically alter nuclear or cytoplasmic concentrations of the *de novo* DNA methyltransferases or global DNA methylation in human PBMCs. Conversely therefore, large transient elevations in circulatory inflammatory markers associated with longer duration exercise may indeed be one of the most important regulators of DNMT localisation within the nucleus, however, as to whether these elevations, in plasma IL-6, for example, are merely a downstream consequence of other circulatory changes such as fluctuations in Ca^{2+} homeostasis, remains to be investigated. A tentative implication of this is that PBMCs may be a more useful biomarker of epigenetic change following aerobic exercise of a

longer duration whereby elevations in circulatory inflammatory markers are common, in contrast to eccentric exercise which is generally characterised by a localised inflammatory response.

Thus far, only the effects of inflammatory cytokines on epigenetic regulation have been investigated. However, as previously postulated, there may in fact be a bi-directional relationship whereby exercise-induced epigenetic modifications directly influence inflammatory gene expression, thus explaining the commonly observed reduction in chronic low-grade inflammation with increasing PA or exercise. Furthermore, as to whether training status influences susceptibility to epigenetic change, be it transcriptional changes to DNMTs, or acute changes to promoter methylation causing transient gene upregulation, remain to be investigated.

CHAPTER 7

ACUTE AND CHRONIC CHANGES TO INFLAMMATORY GENE PROMOTER METHYLATION AND DNMT TRANSCRIPTION FOLLOWING AN AEROBIC TRAINING INTERVENTION

7 Acute and Chronic Changes to Inflammatory Gene Promoter Methylation and DNMT Transcription Following an Aerobic Training Intervention

7.1 Introduction

In the previous chapter, it was shown that a single bout of damaging eccentric exercise was unable to elicit changes in nuclear or cytoplasmic concentrations of either *de novo* DNA methyltransferases, nor global DNA methylation. These data support the hypothesis that transient exercise-induced elevations of circulating inflammatory cytokines are likely to be the main, but not the only, regulator of DNMT relocalisation to the nucleus. Unfortunately, thus far, the cause of the previously observed changes in nuclear DNMT concentrations has not been elucidated and poses the question, are these changes due solely to cellular enzyme transport, transcriptional changes, or a combination of both?

As with many bodies of literature in any scientific sub-discipline, it is common to see inconsistent results between studies. Often, this is due to differences in methodologies, including the population of interest. Trained or recreationally active individuals have been utilised in the studies presented thus far in order to try to understand the mechanisms that underlie exercise-induced modifications to the methylome. Of greater practical understanding to the wider scientific community may be the effects of regular exercise in a sedentary population on the DNA methylation status of genes that are commonly associated with chronic inflammation. As postulated, the relationship between inflammation and epigenetic modification is complex; inflammatory cytokines appear to elicit significant alterations in DNA methylation-regulatory machinery, however, alterations in DNA methylation are also able to cause transient changes in the expression of inflammatory genes. Thus far, the studies within this thesis have focussed specifically on the role of inflammatory mediators on epigenetic modification and not the other direction of the relationship.

Physical inactivity is not only associated with a significantly increased risk of stroke, atherosclerosis, and T2DM (Booth and Lees, 2012), but also, elevations in circulating inflammatory markers in a variety of populations. Physical inactivity has previously been associated with higher plasma CRP in adults (Abramson & Vaccarino, 2002; Autenrieth et al., 2009; Ford, 2002; Henson et al., 2013; León-Latre et al., 2014), whilst time spent watching television was also associated with CRP in children (Gabel et al., 2015). In addition, sedentary behaviour has been correlated with elevated plasma IL-6 (Autenrieth et al., 2009; Henson et al., 2013) and TNFα (Allison et al., 2012). Furthermore, it is postulated that the increased risk of various cancers associated with inactive behaviour is mediated in part by inflammatory mechanisms (Lynch, 2010).

From a non-epidemiological perspective, experimentally-induced physical inactivity in the form of nine days of bed rest is not only able to induce insulin resistance, but is also able to alter the expression of over 4,500 genes in the skeletal muscle of healthy young men. Differential expression of a number of inflammatory pathways was reported, demonstrating how swiftly and dynamically gene expression can be manipulated by activity level (Alibegovic et al., 2010).

Similarly, increasing the amount of exercise results in an attenuation of this chronic inflammatory state, thereby reducing disease susceptibility (Gleeson, Bishop, Stensel, Lindley, Mastana, & Nimmo, 2011). A number of mechanisms have been postulated to mediate the anti-inflammatory effects of exercise such as augmented anti-inflammatory cytokines production, reduced fat mass, and attenuated Toll-like receptor expression on monocytes and macrophages. The effects of exercise on inflammatory gene expression must not be underestimated however. Differential expression of 63 genes in peripheral blood, many involved in inflammatory and immune responses, was reported following 17 weeks of half-marathon training in a previously sedentary sample of men (Vance et al., 2014). Similarly, a shorter and lower intensity training period, specifically, eight weeks of 10,000 steps per day for three days per week, was able to significantly augment peripheral blood leukocyte *IL4* expression and attenuate *IL6* expression (Yakeu et al., 2010). These data were corroborated by another study that utilised a 24 week training period, whereby serum IL-6 concentrations fell, in addition to upregulation of *IL4*, which is known to be an inhibitor of IL-6 (Thompson et al., 2010). IL-10 is another inhibitor of IL-6 production (Hempel et al., 1995) and has been shown to be significantly upregulated, at both mRNA and protein level, following eight weeks of resistance training in PBMCs of healthy elderly participants (Rodriguez-Miguelez et al., 2014). Singular bouts of exercise are also able to elicit a number of transient transcriptional changes of inflammatory genes, with regular training able to attenuate this acute upregulation; eight weeks of eccentric training in an elderly population was sufficient to attenuate acute *NFκB* activation and *IL6* upregulation that was observed following a single bout performed prior to the training period (Jiménez-Jiménez et al., 2008).

The association, therefore, between a more physically active lifestyle and reduced inflammation, both at a transcriptional and circulatory level, is well established. Given the role of epigenetic modification in the regulation of gene expression, in addition to lifestyle-induced alterations to the epigenome, a logical postulation would be that regular exercise is able to attenuate inflammation *via* epigenetic mechanisms.

7.1.1 IL-6

One of the most commonly used biomarkers for systemic inflammation associated with inactivity is IL-6, possibly as it is associated with physical inactivity to a greater extent than other cytokines (Fischer, Bernsten, Perstrup, Eskildsen, & Pedersen, 2007). Furthermore, physical activity exerts a significant effect on IL-6 levels both directly, and indirectly via changes to body composition

(Fitzgerald, Macey, & Brecht, 2012). On a transcriptional level, 10 weeks of knee extensor training was able to attenuate contraction-induced increases in skeletal muscle *IL6* mRNA, although resting levels remained unaltered (Fischer, Plomgaard, Hansen, Pilegaard, Saltin, & Pedersen, 2004). Within PBMCs however, trained swimmers were found to exhibit significantly greater *IL6* mRNA levels at rest compared with controls. Despite appearing paradoxical at first due to regular exercise's ability to attenuate circulating concentrations of IL-6 protein (El-Kader, 2010; Jankord & Jemiolo, 2004; Straub, Tankó, Christiansen, Larsen, & Jessop, 2008), this finding was suggested to be due to the protective, anti-inflammatory role of IL-6 within leukocytes, particularly, IL-10 and IL-1ra upregulation (Capomaccio et al., 2008).

These data demonstrate that regular exercise is closely linked to systemic and transcriptional changes to the *IL6* gene. Most work undertaken in the exercise epigenetics discipline, however, has focussed on the effects of IL-6 protein on DNA methylation, but very little research has studied epigenetic regulation of the *IL6* gene itself, particularly following exercise.

Elevated *IL6* promoter methylation has been reported in peripheral blood of obese Korean women (Na, Hong, Lee, Kim, & Kim, 2015). Similarly, positive associations between weight and DNA methylation at a number of CpG sites was reported in obese and lean control groups, in contrast to the negative correlation observed in T2DM sufferers (Aumueller, Remely, Baeck,Hippe, Brath, Haslberger, 2015). CpG hypomethylation has also been reported in PBMC samples of RA patients, which was related to *IL6* mRNA (Nile et al., 2008). With these data in mind, it seems a plausible supposition that regular exercise would be able to significantly alter *IL6* methylation, thus explaining previously observed transcriptional changes, resulting in an attenuation of chronic low grade inflammation. The pleiotropic nature of IL-6, combined with the inconsistency of transcriptional and epigenetic changes discussed thus far, makes it difficult to hypothesize the precise direction of epigenetic change within the promoter region of this gene.

7.1.2 NF-κB2

Another pathway of key importance in chronic inflammation is mediated by the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is activated by metabolic stress, in addition to pro-inflammatory cytokines such as IL-1 and TNFα (Lawrence, 2009). Upon activation, NF-κB dissociates from the inhibitory molecule IκB, followed by nuclear translocation. The protein then binds to DNA sequences in order to upregulate a number of genes involved in inflammation, immunity and metabolism (Tornatore, Thotakura, Bennett, Moretti, & Franzoso, 2012). Consequently, chronic overactivation of NF-κB is associated with insulin resistance and T2DM (Kramer & Goodyear, 2007). Additionally, aberrant methylation patterns of genes involved in the NF-κB signalling cascade have been associated with celiac disease (Fernandez-Jimenez et al., 2013) and SLE (Zhao et al., 2014).

As already discussed briefly, exercise can acutely upregulate *NFκB* (Jiménez-Jiménez et al., 2008), which has been corroborated by Vider et al. (2001); one hour of running at 80% of $\dot{V}O_{2max}$ was able to elicit transient NF-κB activation in peripheral blood leukocytes. Research also exists that supports this relationship in rat skeletal muscle (Ho et al., 2005; Ji, Gomez-Cabrera, Steinhafel, & Vina, 2004). In the context of regular exercise, 12 weeks of treadmill training was able to elicit to a 44% increase in IκK phosphorylation in rat soleus muscle, as well as a 16% and 22% attenuation of *IκBα* expression in *plantaris* and *soleus*. These findings indirectly infer NF-κB activation, highlighting a possible mechanism by which the reported elevation of *SOCS3* expression may then cause *IL6* upregulation (Spangenburg, Brown, Johnson, & Moore, 2006). *Vastus lateralis* muscle biopsies taken from T2DM patients exhibited overactivation of the NF-κB pathway due to a 60% decrease in IκBβ protein compared with controls, which also correlated with insulin resistance (Sriwijitkamol et al., 2006). Following eight weeks of aerobic exercise however, IκBα and IκBβ protein levels were elevated so that they were comparable with control subjects, concomitantly with a 40% attenuation of muscle TNFα concentrations and improved insulin sensitivity. Thus, regular exercise in a clinical population is able to reverse chronic inflammatory pathways, although this study did not clarify the underlying genetic and epigenetic changes that mediated this. Unlike *IL6*, *NFκB,* specifically the *NFκB2* isoform, has been shown to be regulated by exercise training-induced epigenetic modifications in peripheral blood following six months of walking exercise (Zhang et al., 2015), but this interaction has not been examined in PBMCs or following a shorter, higher intensity training programme. The study utilised an elderly sample, however it was not elucidated whether the observed hypermethylation of *NFκB2* contributed to attenuation of the age-related inflammatory state. Therefore, the use of a younger, but still sedentary, study sample would aid in the clarification of whether exercise is able to ameliorate the inflammatory state directly via downregulation of *NFκB2*.

7.1.3 ASC

ASC, a protein that contains an N-terminal pyrin domain and a C-terminal caspase-recruitment domain, is involved in NF-κB activation, and thus, plays an important role in pro-inflammatory signalling pathways (Masumoto et al., 2003). The protein is able to trigger apoptosis upon overexpression, and is susceptible to methylation-induced silencing (Sarkar, Duncan, Hart, Hertlein, Guttridge, & Wewers, 2006). A plethora of literature confirms that aberrations in *ASC* methylation are linked with the pathogenesis of ovarian cancer (Akahira, Sugihashi, Ito, Niikura, Okamura, & Yaegashi, 2004), human melanoma (Guan et al., 2003), renal cell carcinoma (Liu et al., 2015[b]), invasive ductal breast carcinoma (Mirza et al., 2007), glioblastoma (Stone, Bobo, Brat, Devi, Van Meir, & Vertino, 2004), and experimentally induced autoimmune enceophalomyelitis (Shaw, Lukens, Burns, Chi, Maureen, McGargill, & Kanneganti, 2010). In the context of exercise, the study conducted by Nakajima et al. (2010 - section 1.6.2.4.2) demonstrated that while *ASC* was

hypomethylated in the elderly group, a six month walking programme was able to attenuate this age-related decline in methylation. It would have been of great interest to clarify whether this also attenuated systemic markers of inflammation, however, the study does still provide a plausible mechanism by which exercise-induced epigenetic changes can reverse 'inflamm-aging'.

To summarise, all three genes are involved in chronic inflammatory pathways and disease pathogenesis, have been documented to be epigenetically modified, and susceptible to transcriptional and/or epigenetic changes following exercise. It remains to be elucidated whether *IL6* promoter methylation is altered following exercise training using a sedentary sample, or whether the reported training-induced changes in *ASC* and *NFκB2* methylation are reproducible in a younger population.

In addition to the potential for exercise-induced modifications to the methylome which may reverse unhealthy, inactivity-associated expression profiles of genes involved in chronic inflammation, it is also fair to claim that there is a considerable paucity of research regarding the role of transcriptional alterations in DNMT genes which may underpin these adaptations. A number of studies utilising rodents have demonstrated this (section 1.6.1), however, there is very little research using humans. Only one study was found upon a thorough literature search; three hours of cycling at 60% of $\dot{V}O_{2max}$ was able to significantly attenuate *DNMT3B* mRNA by 50% immediately post-exercise (Laye & Pedersen, 2010). This gap in the literature appears worthy of further research attention.

7.1.4 Aims

The aims of the present study, therefore, were to investigate whether a progressive seven week moderate intensity aerobic training programme elicits significant changes in methylation of *IL6, NFκB2*, and *ASC*, around the respective transcription start sites. It was hypothesized that *ASC* and *NFκB2* methylation would be elevated following the intervention, however, the *IL6* promoter may become hypomethylated in this region, which would be consistent with the previously reported elevation of basal *IL6* mRNA level associated with training status (Capomaccio et al., 2008).

From a more mechanistic standpoint, investigation into how expression of DNMTs may be chronically altered following the intervention, and whether any chronic changes will affect the acute responsiveness to a single maximal bout, will aid in the elucidation of data thus far.

7.2 Methods

7.2.1 Participants

Sedentary, defined in this case as participating in less than one hour of organised exercise per week for the previous six months, but otherwise healthy men between the ages of 25 and 40 were recruited by advertising materials posted around Northumbria University, emails sent to Northumbria University students and staff, in addition to Newcastle and Gateshead council staff newsletters. Participant demographic information is provided in table 7.1. 20 participants were recruited, and randomly split into two groups; an exercise group and a control group. During the course of the study, two members of the exercise group (ExG) and one member of the control group (CoG) dropped out due to time commitments.

7.2.2 Sample Size Estimation

As with all other studies presented with this thesis, sample size was calculated using a spreadsheet accessed from the Boston University and Boston Medical Centre Research Compliance website ("Sample-Size Calculations", 2014) (see appendix E).

7.2.3 Data Collection

7.2.3.1 Initial Screening

Upon confirmation that each participant fit the inclusion criteria of the study, they were contacted to verbally confirm that they were not suffering from any major conditions or musculoskeletal injuries that may contraindicate their ability to participate. They then attended a screening session whereby they also completed a Physical Activity Readiness Questionnaire (PAR-Q) (appendix F) to ensure that they were healthy enough to begin a new exercise regime. Anyone that answered yes to any of the questions were informed that they were to confirm with their General Practitioner that the programme was safe for them to engage in. Following a rest period of at least 10 minutes, resting BP was measured on the right arm using an automatic BP monitor. Two measurements were taken five minutes apart, with the mean value calculated for both. Finally, they were asked to complete a mdFFQ, outlined in section 2.2.3.

7.2.3.2 Habitual Physical Activity and Dietary Assessment

For the following seven days, participants were asked to record their daily food and drink intake in as much detail as possible in a food record diary that was provided (appendix G). Any participants that did not own electronic kitchen scales were provided with some so that they were able to accurately weigh their food. Food diaries were analysed using Nutritics Professional Nutrition

Analysis software (Dublin, Ireland). For the same period of time, each participant wore a tri-axial accelerometer (Actigraph GT3X+, Florida, USA) on their dominant hip. Participants were asked to wear the accelerometer for the entire seven day period, removing only to shower or bathe. Prior to the calculation of physical activity energy expenditure using the Freedson VM3 combination algorithm (Sasaki, John, & Freedson, 2011), age, sex, height, and weight was entered into the Actilife software for each participant. Sixty second sampling epochs were collected at a 60Hz sample rate. Participants were instructed to maintain their usual dietary habits and physical activity habits during the assessment period and for the entire period of the intervention, as to not elicit any changes due to calorie restriction or excess, or significant changes to nutrient intake.

7.2.3.3 Main Trial

Prior to the first testing session, participants were asked to abstain from caffeine, alcohol and the use of NSAIDs for 24 hours prior to each visit, and until the final blood sample was taken at 24 hours post-exercise. Participants were also instructed to not engage in any exercise other than minimal habitual physical activity for the 72 hours prior to participation. On the day of the first testing session, participants arrived having fasted for at least two hours. All visits occurred between 10:00 and 15:00, with the time and day replicated at the post-training testing session for each participant.

Firstly, height, weight, hip circumference and waist circumference were measured using standard procedures outlined in chapter 2 (section 2.2.1). Following this, a 30 ml venous blood sample was taken from a vein in the antecubital fossa region of the forearm using standard procedures outlined in chapter 2 (section 2.2.4). 10 ml was immediately centrifuged, the plasma aspirated into microcentrifuge tubes, and store at -80 ^{o}C for until further analysis. The remaining 20 ml of whole blood was used in order to isolate PBMCs using the procedure outlined in section 2.3.4.

An incremental treadmill walking test to volitional exhaustion, defined by rating of perceived exertion (RPE) on a standard Borg scale, was utilised. The modified Bruce protocol (table 7.1) was used as it was developed for sedentary and clinical populations (Bruce, Kusumi, & Hosmer, 1973; Hill & Timmis, 2002). A maximal test was preferred to a submaximal test as to mimic previous research (Barrès et al., 2012). An online breath by breath gas analyser (Metalyzer 3B, Cortex, Germany) measured inspired and expired gases. RPE and HR, via short range telemetry (Polar RS400, Finland), were recorded during the last 10 seconds of each minute. The test was considered maximal if the following criteria were fulfilled; a change in $\dot{V}O_2$ of less than 2 ml·kg·min^{-1} was observed despite an increase in intensity, a minimum respiratory exchange of 1.15, or a HR greater than 90% of age predicted maximum. Due to the sedentary nature of the study population, some participants reached exhaustion prior to a plateau in $\dot{V}O_2$ due to local fatigue in the legs; however, all participants reached at least 90% of their age-predicted maximum HR and a RER of 1.15. The values were, therefore, deemed to be $\dot{V}O_{2peak}$.

Table 7.1 - Modified Bruce protocol.

Stage	Minutes	Gradient (%)	Speed ($km \cdot h^{-1}$)
i	3	0	2.7
ii	3	5	2.7
1	3	10	2.7
2	3	12	4.0
3	3	14	5.4
4	3	16	6.7
5	3	18	8.0
6	3	20	8.8
7	3	22	9.6

A further 10 ml blood sample was taken immediately upon cessation of exercise. The participant then remained in a rested state in the laboratory with *ad libitum* access to water until a final sample for that day was taken two hours following cessation of exercise. As discussed, the participants continued all restrictions until the next day, including a two hour fast, when a final blood sample was taken 24 hours following completion of the maximal exercise test.

Participants were then randomly assigned to control (CoG) or exercise groups (ExG). The control group were instructed to maintain their usual activity and dietary habits during the entire study period. The exercise group were provided with a progressive seven week training plan as previously utilised by Barwell et al. (2008) and Moran et al. (2011). Participants were asked to walk or jog on a treadmill at a speed or incline that would allow them to maintain their HR between 65 and 80% of their individual age-predicted maximum HR. They were provided with a trackable HR monitor (Polar RS400) in order to do this. Participants were required to perform one session each week in the laboratory so that the HR data could be downloaded to ensure compliance to the programme. The full details of the training programme are provided below in figure 7.1 and table 7.2.

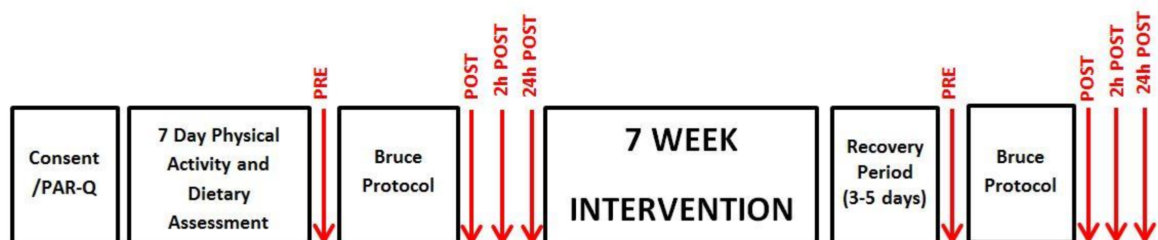

Figure 7.1 *- Schematic of training study timelime (red arrows indicate blood samples).*

Following the intervention and a three to five day rest period, participants returned to the lab to repeat all testing measures as before. They were informed to replicate the food they consumed the day before and the morning of the pre-intervention trial.

Table 7.2 *- Seven week training intervention.*

Week	Session Details
1	3 x 30 mins
2	2 x 30 mins; 2 x 45 mins
3	3 x 30 mins; 2 x 45 mins
4	5 x 45 mins
5	3 x 45 mins; 2 x 60 mins
6	5 x 60 mins
7	5 x 60 mins

7.2.4 Lab Analyses

7.2.4.1 Flow Cytometry

Flow cytometry was used in order to ensure that any observable changes in DNA methylation were not due to changes to the fractional composition of the PBMC samples.

PBMC samples were thawed quickly in a waterbath at 37 ^{o}C and washed using 1x PBS by centrifugation at 400*g for five minutes. Supernatants were discarded and cells were counted using a Casey TT cell counter and analyser system (Roche, Basel, Switzerland). Cells were diluted with 1x PBS to yield $20*10^{6}\cdot ml^{-1}$. 50 µl of cells ($1*10^{6}\cdot ml^{-1}$) were then incubated at room temperature for 15 minutes in the dark with the antibody composition outlined in table 7.3 in order to identify T, B, and NK cell population. All antibodies were purchased from eBioscience (Affymetrix, Chesire, UK) unless indicated. Following incubation, cells were washed with 1x PBS and centrifuged as before. In order to exclude dead cells from the analysis, the stained PBMCs were then resuspended in 1 $ug\cdot ml^{-1}$ of DAPI solution (BD Biosciences, Oxford, UK) and incubated for a further 10 minutes at room temperature in the dark before being acquired on a BD FACSCanto II flow cytometer (BD Biosciences, Oxford, UK). Data were analysed using BD FACSDiva software.

Table 7.3 *- Details of antibodies used for flow cytometric analysis.*

Cell Surface Receptor	Clone	Flourochrome	Laser	EM-max[a]
CD19	MB19-1	FITC	488	525
CD3	OKT3	PE	488	575
CD16[b]	3G8	PerCP-Cy5.5	488	695
CD8	RPA-T8	PE-Cy7	488	785
CD4[b]	RPA-T4	APC	633	660
CD56	CMSSB	APC-H7	633	785

[a] *Maximum emission wavelength (nm)*

[b] *BD Biosciences, Oxford, UK*

7.2.4.2 Bisulfite-conversion of DNA

Cells were thawed, washed and counted using the procedure outlined in chapter 2. Cells were then diluted in R0 medium to yield $4*10^5 \cdot ml^{-1}$. In individual wells, 250 µl of cells (i.e. $1*10^5$ cells) were incubated for four hours at 37 °C with 100 µl of exercise-conditioned plasma from each timepoint and 150 µl of R0 medium. In contrast to previous chapters, participants' own cells were used instead of a standardised cell sample in all conditions. Therefore, pre-training exercise-conditioned plasma was used to stimulate pre-training cell samples, and post-training exercise-conditioned plasma was used to stimulate cell samples isolated following the programme. Similarly, in the CoG, exercise-conditioned plasma from the first bout was used to stimulate cells isolated at the first visit and so forth for the subsequent bout, despite a lack of a training intervention in between. Separately, a sample of PBMCs isolated from a resting participant that was not involved with the study but met all inclusion criteria were stimulated with various cytokines in duplicate; 10 $mg \cdot ml^{-1}$ stock solutions of IL-6, TNFα and IL-1β were diluted with R0 medium to yield 1 $pg \cdot ml^{-1}$ ('Low') and 10 $pg \cdot ml^{-1}$ ('High') concentrations. These cytokines were selected due to previous literature that reported their influence over DNA methylation and regulatory enzymes, in addition to their key roles in inflammatory processes.

After the incubation period, cells were again washed with 1x PBS by centrifugation, then DNA was bisulfite converted (bsDNA) (EpiTect Fast Bisulfite Conversion Kit, Qiagen, Germany). Bisulfite treatment of DNA results in all unmethylated cytosines being converted to uracils, while methylated cytosines are protected from this modification. Briefly, the procedure involved preparation of DNA from cell samples, bisulfite-mediated conversion of unmethylated cytosines with a highly concentrated bisulfite solution, binding of single-stranded DNA to a spin column membrane, repeated washing, desulfonation of DNA bound to the column membrane, further washing, and finally, elution of the converted DNA. The bisulfite conversion itself was performed using 0.2 ml PCR tubes and thermal cycling (CFX96, BioRad, California, USA) with the following

settings: five minutes at 95 ºC, 10 minutes at 60 ºC, five minutes at 95 ºC, and 10 minutes at 60 ºC. BsDNA was eluted in 20 µl of elution buffer and stored at -20 ºC for later analysis of DNA methylation.

7.2.4.3 Methylation Sensitive High Resolution Melting

Methylation sensitive High Resolution Melting (msHRM) is a PCR-based method for quantifying locus-specific DNA methylation changes that amplifies templates regardless of methylation status, in contrast to methylation-specific PCR which preferentially amplifies methylated or unmethylated templates and cannot, therefore, quantitatively distinguish between low and high levels of methylation (Balic et al., 2009). MsHRM is able to detect 1% methylated DNA in a background of unmethylated DNA, indicating that it is a sufficiently sensitive measure (Krypuy, Wojdacz, & Dobrovic, 2008). Amplified methylated DNA results in a higher melting temperature than unmethylated, and vice versa, resulting in differential melt curves.

Appropriate primers were first designed and tested. Using the 1000 Genomes browser ("1000 Genomes", 2015), sequences that included the first exon and the 1000 bp flanking region were identified in the *IL6*, *NFκB2,* and *ASC* genes. These sequences were pasted into Methprimer, a program used for designing PCR primers for methylation analysis (Li and Dahiya, 2002). A number of potential assays were selected and ordered from IDT (Integrated DNA Technologies, Iowa, USA). All primer pairs were checked for product specificity and dimerisation using gel electrophoresis (BioRad PowerPac HC, California, USA). Optimal annealing temperatures were also testing using gel electrophoresis (see appendix H).

All utilised primer pairs overlapped a CpG island in the first exon or promoter region (see mapped sequences in appendix I), given that first exon methylation is also linked with transcriptional silencing (Brenet et al., 2011). Furthermore, EpiTect HRM PCR guidelines state that PCR product lengths should be between 70 and 200 bps, and primer pairs should have similar annealing temperatures. In line with these recommendations, the amplicons of the selected assays were between 110 and 180 bps, and annealing temperatures of the primer pairs within 2 ºC.

5 µl of HRM-PCR master mix, 0.75 µl of 10 µM forward and 0.75 µl of 10 µM reverse primer, 2 µl of bsDNA, and 1.5 µl RNase-free water were added to each well of a 384-well plate, with a total well volume of 10 µl. Methylated and unmethylated bsDNA controls (EpiTect PCR Control DNA set, Qiagen, Germany) (100, 50, 25, 12.5, 6.25, 3.125, 0% methylated DNA) were included, in addition to blank controls which did not include any DNA. Each sample or control was assayed in triplicate using the Roche LightCycle 480 qPCR System (Roche, Basel, Switzerland) (Krypuy et al., 2008). PCR settings are outlined in table 7.4.

Table 7.4 *- qPCR Settings for High Resolution Melting Analysis.*

	No. Cycles	Temperature (^oC)	Hold (mins:secs)
Pre-incubation	1	95	05:00
Amplification	50	-	-
	-	95	00:10
	-	55	00:30
	-	72	00:10
HRM	1	-	-
	-	60	00:01
	-	58*	Continuous increase
Cooling	1	40	00:01

**NFκB2* ran at 55°C

7.2.4.4 Isolation and Purification of RNA

Due to limitation of resources only pre- and post-training cells isolated from the ExG were incubated in the presence of pre, post, and 2h-post exercise-conditioned plasma for four hours at 37 °C. Cells were then diluted in R0 medium to yield $4*10^6 \cdot ml^{-1}$. 250 µl of cells (i.e. $1*10^6$ cells) were incubated for four hours at 37 °C with 100 µl of exercise-conditioned plasma from each timepoint and 150 µl of R0 medium.

After the incubation period, cells were again washed with 1x PBS by centrifugation, then RNA isolated and purified (RNeasy Mini Kit, Qiagen, Germany). Briefly, the protocol involved homogenisation of lysate using centrifugation of QIAshredder spin columns, transferral of the sample to the provided MinElute spin columns and centrifugation, complete removal of DNA using RNase-free DNase set (Qiagen, Germany), and further centrifugation of spin columns. RNA was eluted in 50 µl of elution buffer and aliquoted in 0.2 ml PCR tubes for storage at -80 °C.

7.2.4.5 Reverse Transcription of RNA to cDNA

Prior to reverse transcription of RNA to complementary DNA (cDNA), concentrations of RNA were quantified using the Nanodrop ND1000 spectrophometer (ThermoFisher Scientific, Massachusetts, USA) which was first calibrated using CF-1 fluid (see figure 7.2). All RNA samples were between 1 and 4 $ng \cdot ml^{-1}$. A 14 µl reaction mix, including 2 µl of gDNA wipeout buffer, was required, therefore, 12 µl (12 to 48 ng of RNA) was utilised for reverse transcription (QuantiTect Reverse Transcription, Qiagen, Germany). The mix was incubated at 42 °C for two minutes then reverse transcriptase added followed by further incubation at 42 °C for 20 minutes and 95 °C for three minutes to inactivate the reverse transcriptase. Reactions were then stored at -20 °C.

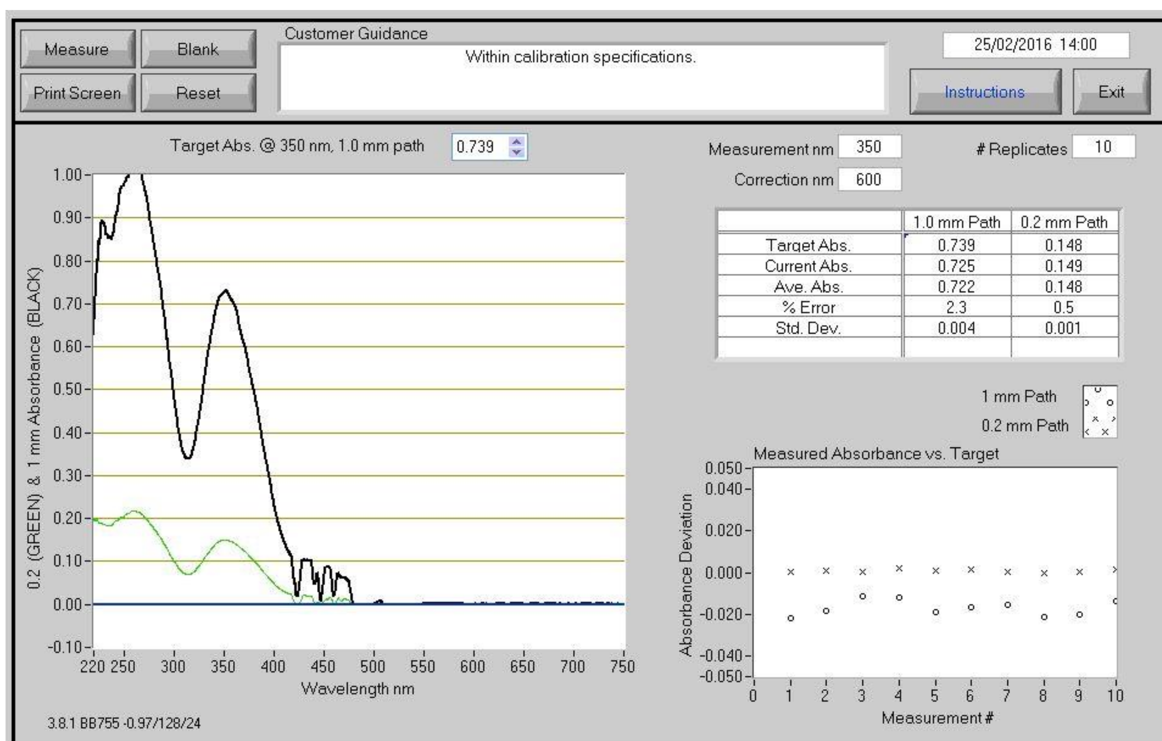

Figure 7.2 *- ND1000 spectrophotometer calibration check.*

7.2.4.6 DNMT3A and DNMT3B Gene Expression

Using the 1000 Genomes browser ("1000 Genomes", 2015), protein coding transcripts were identified for *DNMT3A* and *DNMT3B*. These transcripts were then cross-referenced with those recognised by the National Centre for Biotechnology Information (NCBI) (appendix J). Using the Roche assay design centre, a common assay was designed which includes all coding transcripts, in addition to primers for each individual protein coding transcript. All primers were selected to be intron spanning to avoid amplification of genomic DNA. All primers are presented in appendix J; duplicate sequences were eliminated immediately, and using cDNA pooled from all samples to be tested, qPCR was used to check that each pair successfully amplified the cDNA. The common assay for *DNMT3A* successfully amplified the cDNA. None of the *DNMT3B* primers were successful. *RPLP0* was used as a control gene (Wang et al., 2012[a]). 2.5 µl of ABsolute blue qPCR mix (Thermo-Fisher Scientific, Massachusetts, USA), 0.5 µl of 10 µM forward and 0.5 µl of 10 µM reverse primer, 0.15 µl of a relevant Universal ProbeLibrary probe (Roche, Basel, Switzerland), 0.35 µl RNase-free water, and 1 µl of cDNA were added to each well of a 384-well plate, with a total well volume of 5 µl. Dilutions (1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64 or 50, 25, 12.5, 6.25, 3.125, 1.5625, 0% cDNA) of pooled cDNA samples were included as controls on each 384-well plate. Each sample or control was assayed in triplicate using the Roche LightCycler 480 qPCR System (Roche, Basel, Switzerland) (Krypuy et al., 2008). PCR settings are outlined in table 7.5.

***Table 7.5** - qPCR Settings for analysis of gene expression.*

	No. Cycles	Temperature (°C)	Hold (mins:secs)
Pre-incubation	1	95	10:00
Amplification	55 - -	- 60 95	- 01:00 00:15
Cooling	1	40	00:30

7.2.5 Statistical Analysis

Data was analysed using IBM SPSS Statistics version 22. Prior to performing parametric testing of the data, the Shapiro-Wilk test of normality was utilised. Non-normally distributed data was subsequently $\log_{10}$ transformed. Levene's Test for Equality of Variances and Independent Samples T Tests were used to analyse baseline differences between the exercising and control groups. Paired Samples T Tests were used to test for significant differences between pre- and post-intervention data and cytokine-stimulated cell data.

For msHRM analysis, Roche Gene Scanning software (Roche, Basel, Switzerland) was utilised. Raw melt curves were normalised for fluorescence intensity by calculation of two normalisation regions before and after the major fluorescence decrease representing the melting of the PCR product. A temperature shift was applied to correct for different C_t (threshold cycle) values between samples. The software automatically deselects which did samples did not function correctly; however, this was also performed manually, with any samples that exhibited a vastly different amplification curve also removed from further analysis. Normalised and temperature shifted melt curve data was then exported to Microsoft Excel (Office 2016, Microsoft, USA). For each of the three genes, the temperature at which the 0% methylation standard flattened and where all other methylated DNA samples appear in the correct order was marked. The mean relative signal of each standard at this temperature ± 0.2°C was used in order to generate a standard curve. Linearity of the standard curves was checked using Pearson's correlation coefficient prior to generation of methylation data from the samples. Statistical significance was calculated using a linear mixed model using Bonferonni adjustment for multiple comparisons, as a standard repeated measures ANOVA would exclude a participants' entire dataset due to a single missed datapoint. Samples were considered as hyper- or hypomethylated when the methylation level exceeded the mean methylation level of baseline samples by two times the standard deviation (Naghitorabi, Asl, Sadeghi, Rabbani, Jafarian-Dehkordi & Javanmard, 2013). Mean intra-plate CoVs for *IL6, NFκB2,* and *ASC* were 1.71%, 0.28%, and 0.32%, respectively.

For analysis of gene expression, the $\Delta\Delta C_t$ method was utilised. This is a comparative quantification method using the C_t of the gene of interest and a housekeeping gene, in this instance, RPLP0. The average C_t value was calculated for the three wells, and fold difference in expression, relative to the control, was calculated using $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). Mean intra-plate CoV was calculated 1.23%.

7.3 Results

7.3.1 Pre- and Post-intervention Data

Baseline characteristics of both groups are presented in table 7.6. There were no statistically significant differences between groups at baseline for any variables (age, mass, BMI, waist and hip circumference, waist to hip ratio, systolic or diastolic BP, $\dot{V}O_{2peak}$, energy intake or energy expenditure). According to the American College of Sports Medicine, BP values would define participants as pre-hypertensive (Pescatello, Arena, Riebe, & Thompson, 2014, p.46). Furthermore, BMI would classify participants as overweight, although waist circumference and waist to hip ratio would not place the participants in a high risk classification (Pescatello et al., 2014, p.66).

***Table 7.6** - Mean (± SD) articipant characteristics at baseline (ExG = 8; CoG = 9).*

Variable	ExG (Mean ± SD)	CoG (Mean ± SD)
Age (years)	32 (± 4)	29 (± 4)
Stature (cm)	177.7 (± 5.8)	182.8 (± 9.1)
Mass (kg)	89.3 (± 16.6)	86.8 (± 12.5)
Body Mass Index (kg·m^2)	28.2 (± 4.4)	25.9 (± 2.9)
Waist Circumference (cm)	93.9 (± 11.0)	89.9 (± 7.7)
Hip Circumference (cm)	106.0 (± 8.4)	103.6 (± 6.1)
Waist to Hip Ratio	0.88 (± 0.05)	0.87 (± 0.04)
Systolic BP (mmHg)	130.0 (± 11.7)	136.8 (± 7.4)
Diastolic BP (mmHg)	85.5 (± 6.4)	84.8 (± 8.9)
$\dot{V}O_{2peak}$ (ml·kg^{-1}·min^{-1})	43.69 (± 5.59)	47.19 (± 4.72)
Energy Intake (kcal·day^{-1})	1984.3 (± 373.9)	2175.3 (± 277.9)
Energy Expenditure (kcal·day^{-1})	767.5 (± 307.2)	686.2 (± 229.4)

There were no statistically significant differences between groups in terms of habitual dietary intake of the methyl-donors methionine, folate, betaine, and choline.

***Table 7.7** - Estimated habitual dietary intake of methyl-donors.*

Methyl-donor	ExG (Mean ± SD)	CoG (Mean ± SD)
Methionine (mg·day^{-1})	1606.3 (± 579.2)	1633.5 (± 649.8)
Folate (µg·day^{-1})	246.2 (± 169.9)	223.9 (± 111.7)
Betaine (mg·day^{-1})	91.5 (± 46.2)	147.2 (± 77.6)
Choline (mg·day^{-1})	287.2 (± 123.8)	280.8 (± 105.1)

As intended, no significant changes occurred from baseline to follow up in the control group for any of the variables (see table 7.8).

Table 7.8 *- Mean (± SD) control group data pre- and post-intervention period.*

Variable	Pre (Mean ± SD)	Post (Mean ± SD)
Mass (kg)	86.8 (± 12.5)	87.1 (± 12.1)
Body Mass Index (kg·m^2)	25.9 (± 2.9)	26.1 (± 2.9)
Waist Circumference (cm)	89.9 (± 7.7)	90.7 (± 7.4)
Hip Circumference (cm)	103.6 (± 6.1)	104.2 (± 5.8)
Waist to Hip Ratio	0.87 (± 0.04)	0.87 (± 0.03)
Systolic BP (mmHg)	136.8 (± 7.4)	134.6 (± 6.2)
Diastolic BP (mmHg)	84.8 (± 8.9)	84.1 (± 7.2)
$\dot{V}O_{2peak}$ (ml·kg^{-1}·min^{-1})	47.19 (± 4.72)	46.1 (± 4.5)
Energy Intake (kcal·day^{-1})	2175.3 (± 277.9)	1984.9 (± 219.1)
Energy Expenditure (kcal·day^{-1})	686.2 (± 229.4)	590.1 (± 234.5)

Following the exercise intervention, there was a statistically significant reduction in mass, BMI, waist and hip circumferences, and a significant increase in $\dot{V}O_{2peak}$ of 12.2%. As instructed, no significant changes occurred in terms of energy intake and habitual energy expenditure (see table 7.9).

Table 7.9 *- Mean (± SD) exercise group data pre- and post-intervention period.*

Variable	Pre (Mean ± SD)	Post (Mean ± SD)
Mass (kg)	89.3 (± 16.6)	87.2 (± 17.2)*
Body Mass Index (kg·m^2)	28.2 (± 4.4)	25.9 (± 2.9)*
Waist Circumference (cm)	93.9 (± 11.0)	91.2 (± 11.1)*
Hip Circumference (cm)	106.0 (± 8.4)	104.4 (± 7.7)*
Waist to Hip Ratio	0.88 (± 0.05)	0.87 (± 0.06)
Systolic BP (mmHg)	130.0 (± 11.7)	126.9 (± 8.8)
Diastolic BP (mmHg)	85.5 (± 6.4)	80.5 (± 6.8)
$\dot{V}O_{2peak}$ (ml·kg^{-1}·min^{-1})	43.69 (± 5.59)	49.01 (± 7.75)*
Energy Intake (kcal·day^{-1})	1984.3 (± 373.9)	1961.5 (± 499.0)
Energy Expenditure (kcal·day^{-1})	767.5 (± 307.2)	643.7 (± 231.1)

**significantly different from 'pre' ($p < 0.05$).*

Participants were provided with a trackable HR monitor so that they would be able to exercise at the prescribed 65 to 80% of age predicted maximum HR. As shown in table 7.10, the mean HR attained throughout all of the sessions equated to approximately 75% of age predicted maximum HR. Furthermore, throughout the entire intervention period, 95% of sessions were successfully completed.

Table 7.10 *- Mean (± SD) compliance to the prescribed sessions and subsequent recorded heart rate.*

Variable	(Mean ± SD)
Compliance (%)	94.5 (± 2.6)
HR (beats·min^{-1})	140 (± 7)
HR (age predicted max.)	186.8 (± 3.6)
HR (% of age predicted max.)	74.9 (± 3.9)

7.3.2 Flow Cytometric Analysis

Table 7.11 shows that there were no time effects for either group for any of the cell populations that were analysed. A statistically greater percentage of overall lymphocytes was observed in the control group compared with the exercise group at baseline. In both groups at both timepoints, live lymphocytes represent the overall majority of cells present in the PBMC samples, with T cells the most present sub-population.

Table 7.11 *- Mean (± SD) fractional composition of pre- and post-intervention PBMC samples.*

	ExG (Mean ± SD)		CoG (Mean ± SD)	
	Pre	**Post**	**Pre**	**Post**
Lymphocytes	55.2 (± 8.0)	56.3 (± 4.9)	63.4 (± 6.4)*	62.2 (± 7.4)
Single Cells	99.3 (± 0.3)	99.5 (± 0.1)	99.2 (± 0.2)	99.2 (± 0.2)
Live Cells	98.2 (± 0.92)	98.0 (± 2.5)	97.5 (± 1.8)	98.5 (± 0.6)
T cells (CD3$^+$) (% of lymph.)	66.3 (± 9.9)	67.8 (± 10.8)	62.8 (± 13.9)	66.0 (± 13.0)
CD4$^+$ (% of CD3$^+$)	53.7 (± 10.6)	53.9 (±10.4)	51.3 (± 15.0)	46.5 (± 21.5)
CD8$^+$ (% of CD3$^+$)	32.6 (± 8.4)	34.5 (± 9.3)	39.8 (± 17.8)	42.6 (± 21.5)
NK cells (CD3$^-$CD56$^+$) (% of lymph.)	20.9 (± 8.6)	20.6 (± 8.2)	26.8 (± 12.9)	23.2 (± 11.5)
CD56dim (% of NK)	19.3 (± 8.2)	18.7 (± 8.2)	25.2 (± 12.5)	21.6 (± 10.9)
CD16$^+$ (% of CD56dim NK)	78.7 (± 21.9)	85.9 (± 8.5)	80.3 (± 9.1)	74.8 (± 17.1)
CD56bright (% of NK)	1.375 (± 1.3)	1.2 (± 0.4)	0.7 (± 0.4)	0.9 (± 0.7)
CD16$^+$ (% of CD56bright NK)	40.8 (± 18.5)	38.0 (± 14.5)	26.9 (± 18.1)	32.8 (± 11.8)
B Cells (CD3$^-$CD19$^+$) (% of lymph.)	8.6 (± 3.2)	8.6 (± 2.3)	6.2 (± 2.6)	5.6 (± 4.5)

significantly different from 'ExG pre' ($p < 0.05$).

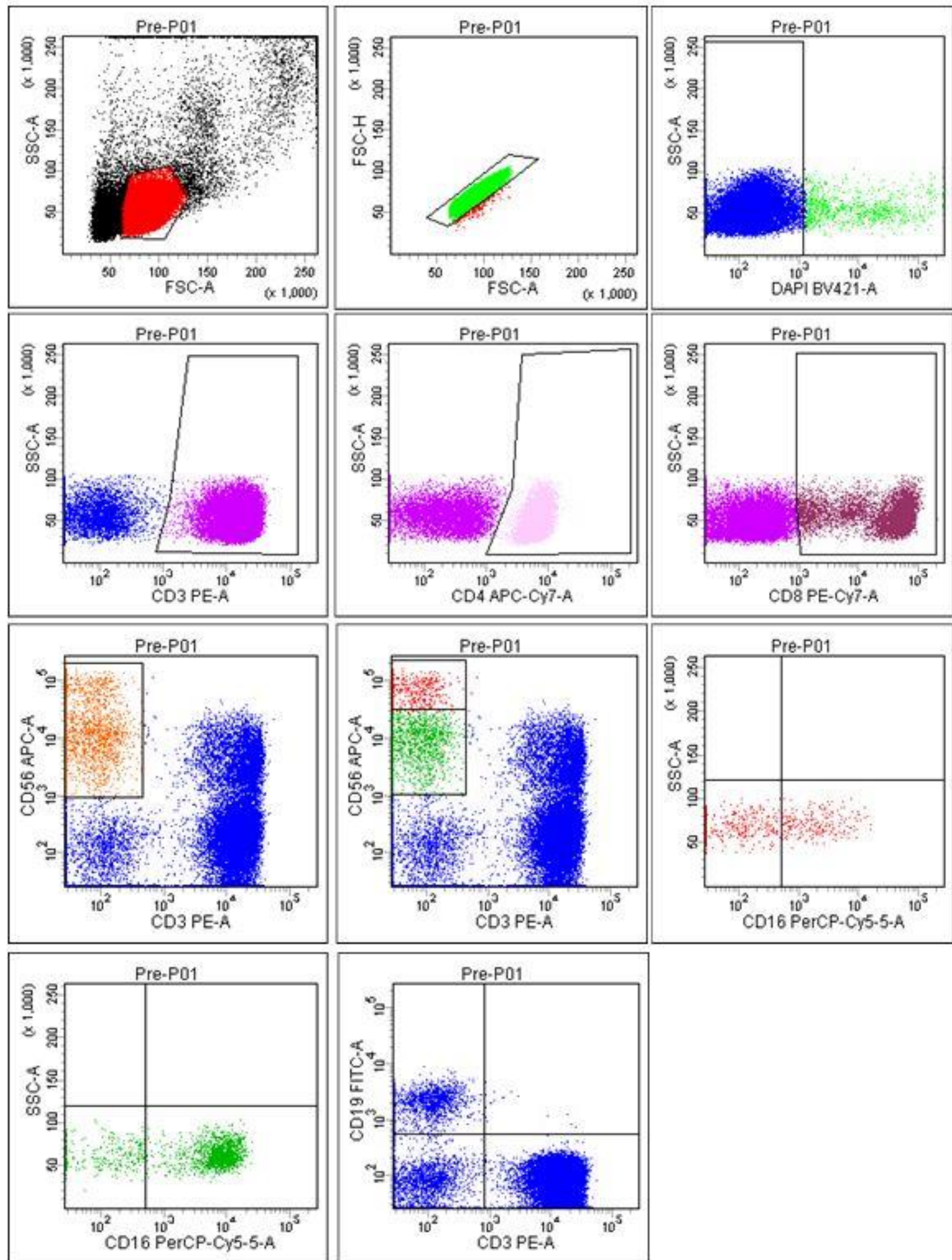

Figure 7.3 *- Example flow cytometry plots from one participant (from left to right, top to bottom): lymphocytes; single cells; live cells; CD3^{+} T cells; CD4^{+} T helper cells; CD8^{+} cytotoxic T cells; total CD3^{-}CD56^{+} NK cells; CD56bright and CD56dim NK cells; CD16^{+} CD56bright NK cells; CD16^{+} CD56dim NK cells; CD3^{-}CD19^{+} B cells.*

7.3.3 Gene-specific DNA Methylation

Pearson's correlation coefficients demonstrate that the standard curves for all genes were highly linear (R > 0.97 for all genes). Appendix K shows the normalised and temperature shifted melt curves for the standards with and without samples for *IL6*, *NFκB2*, and *ASC*.

No statistically significant changes occurred in *IL6*, *NFκB2*, or *ASC* methylation at any timepoint. Data are presented in figures 7.4 to 7.6 (PreTR = Pre-training; PostTR = Post-training. PreEX = Pre-acute exercise; PostEX = Post-acute exercise; 2hEX = 2h post-acute exercise; 24hEX = 24h post-acute exercise).

Unfortunately, all samples including standards on the left side of the *IL6* control sample plate were unsuccessful, suggesting a systematic error with this particular plate. Methylated standard melt curves on the *NFκB2* and *ASC* control sample plates were not sufficient to generate a linear standard curve from which participant sample methylation data could be quantified.

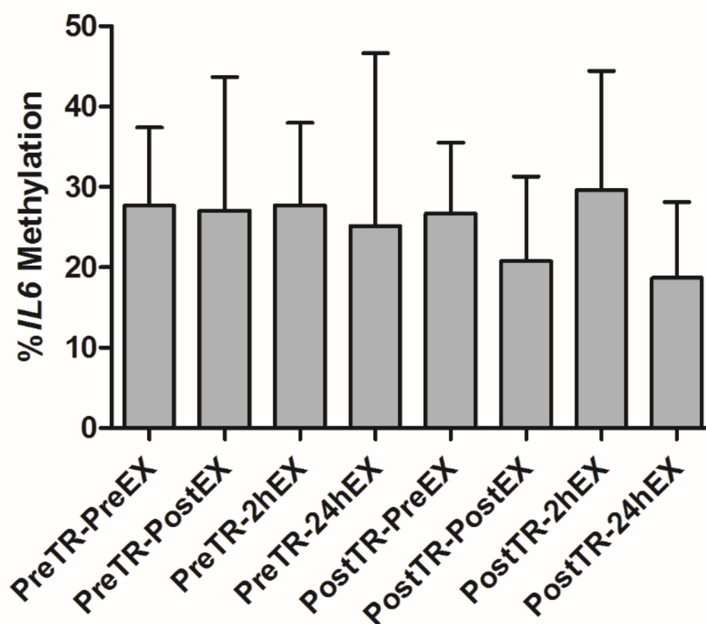

Figure 7.4 *- Mean (± SD) % IL6 promoter methylation at each timepoint.*

10 pg·ml^{-1} ('High') of IL-6 caused a statistically significant attenuation in *IL6* methylation in contrast to a low dose of 1 pg·ml^{-1} ($p = 0.010$). This 9.4% decrease in methylation was twice the standard deviation of the low dose, and thus, can be classified as hypomethylated under the definition outlined in section 7.2.5. 10 pg·ml^{-1} ('High') of IL-1β caused a statistically significant augmentation of *IL6* methylation in contrast to a low dose of 1 pg·ml^{-1} ($p = 0.011$), however, this difference did not exceed the predefined value to be classified as hypermethylated. All other cytokine experiments did not result in significant alterations in gene methylation. Data are presented in figures 7.7 to 7.9.

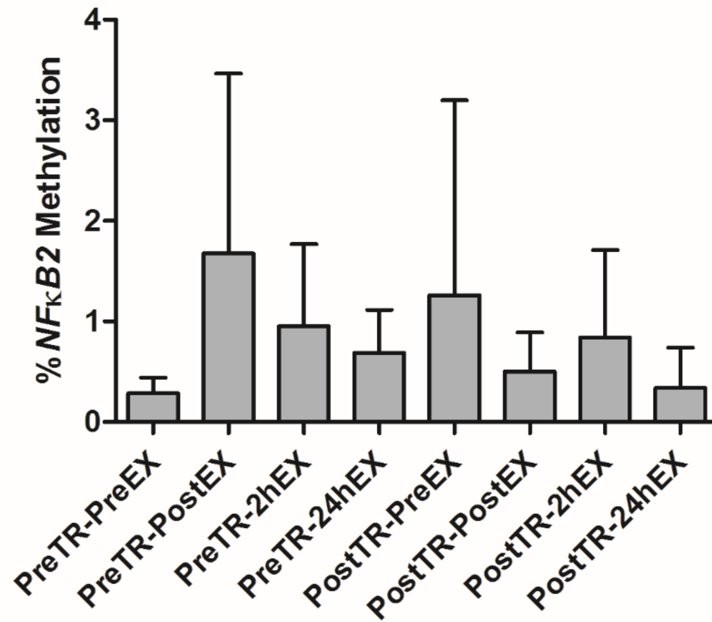

Figure 7.5 *- Mean (± SD) % NFκB2 first exon methylation at each timepoint.*

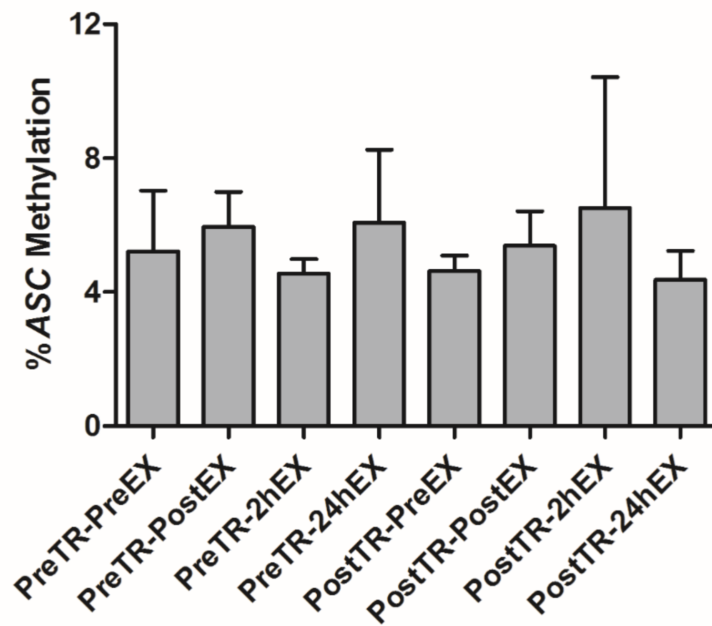

Figure 7.6 *- Mean (± SD) % ASC first exon methylation at each timepoint.*

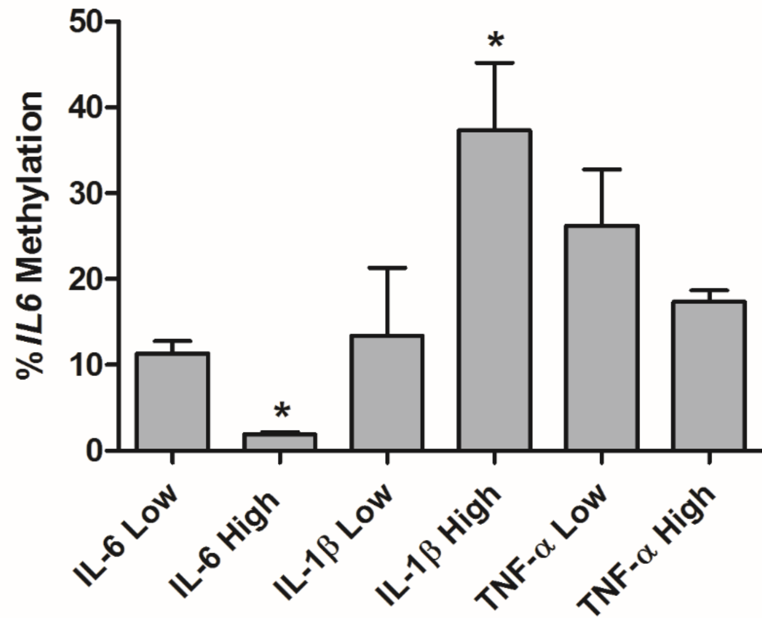

Figure 7.7 *- Mean (± SD) % IL6 promoter methylation following cytokine stimulation. *significantly greater than 'Low' ($p < 0.05$).*

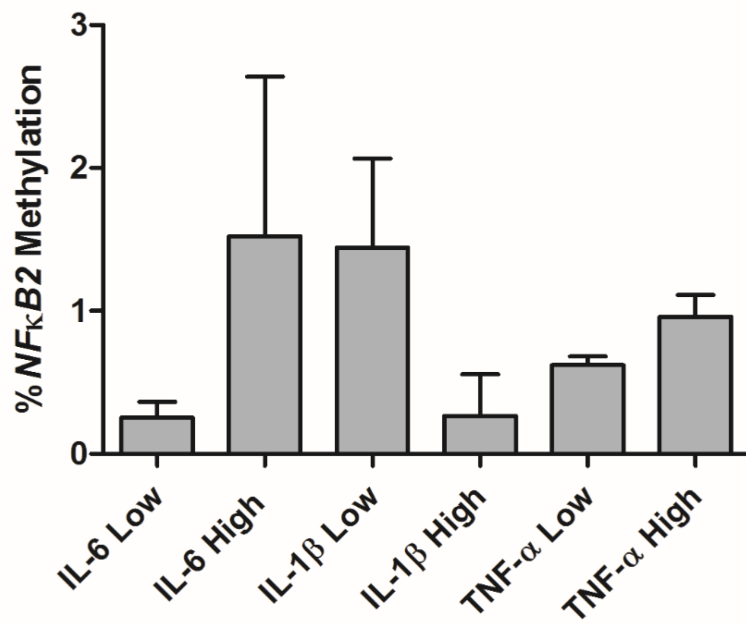

Figure 7.8 *- Mean (± SD) % NFκB2 first exon methylation following cytokine stimulation.*

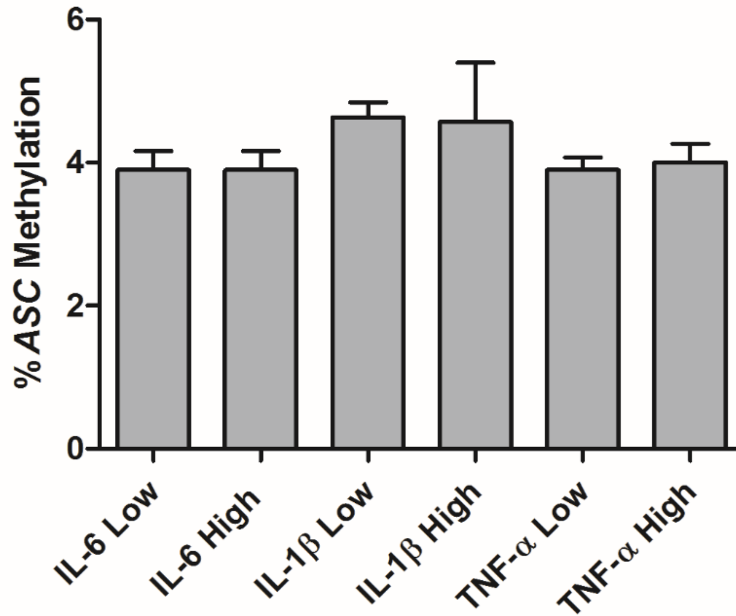

Figure 7.9 *- Mean (± SD) % ASC first exon methylation following cytokine stimulation.*

7.3.4 *DNMT* Gene Expression

No statistically significant changes occurred in expression of *DNMT3A* relative to the control gene *RPLP0* at any timepoint. Data are presented in figure 7.10 (PreTR = Pre-training; PostTR = Post-training. PreEX = Pre-acute exercise; PostEX = Post-acute exercise; 2hEX = 2h post-acute exercise).

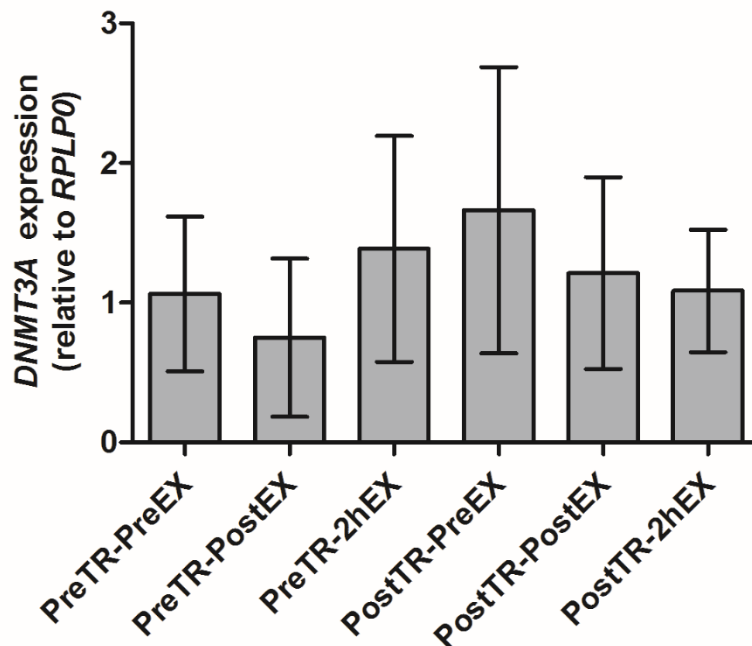

Figure 7.10 *- Mean (± 95% CI) DNMT3A expression relative to control.*

7.4 Discussion

In corroboration with previous studies that have also used the same seven week aerobic exercise intervention, a 12% increase in $\dot{V}O_{2peak}$ was observed (Barwell et al., 2008), in addition to a significant attenuation of body mass, and waist and hip circumferences, similarly to the reported reduction in fat mass by Moran et al. (2011). No significant alterations in promoter or first exon methylation of *IL6*, *NFκB2*, or *ASC* occurred following cell stimulation with exercise-conditioned plasma isolated before and after the exercise intervention or acute maximal bout. Similarly, there was no significant alteration in the expression of *DNMT3A* at any timepoint. *In vitro* stimulation with IL-6 caused significant hypomethylation of the *IL6* promoter, while IL-1β elicited a significant augmentation of methylation, although not classified as hypermethylation by pre-define criteria.

The positive physiological adaptations, combined with the high compliance to the semi-supervised intervention, highlights that this particular intervention was efficacious, and in addition to the progressive nature of the programme, could be an ideal way for sedentary overweight men to improve cardiovascular fitness and body composition. These benefits were not due to changes in increased habitual activity outside of the exercise sessions or changes to dietary (methyl-donor or calorie) intake, therefore, it can be concluded with relative confidence that it was indeed the exercise intervention that elicited these salubrious physiological changes.

Despite this, it was surprising that no significant alterations in DNA methylation occurred. Denham et al. (2015[b]) have demonstrated that as little as four weeks of training can elicit a number of gene-specific methylation changes in leukocytes. With a focus on genes involved in inflammation, Zhang et al. (2015) have previously shown that a regular low intensity exercise programme over six months caused hypermethylation of a number of sites within the *NFκB2* promoter region. Similarly, following the same intervention, Nakajima et al. (2010) demonstrated that exercise was able to cause CpG island hypermethylation in the first exon of the *ASC* gene compared with sedentary controls. There are, however, some clear distinctions between these data and the data presented in this chapter; firstly, although assays were designed for both *NFκB2* promoter and first exon regions in the present study, the latter primers were deemed to be optimal, which contrasts the region analysed by Zhang et al. (2015). This might help to explain why previous studies have clearly shown that exercise can acutely induce *NFκB* activation, and exercise training can attenuate this acute induction of *NFκB* and *IL6*; it is plausible that changes in methylation did indeed occur in regions of these genes associated with transcriptional changes, however, the primers that were utilised did not capture the locus of these modifications. Since protein or mRNA levels were not quantified, it is uncertain whether any functional downstream changes did indeed occur. Additionally, the vast difference in the ages of the study populations clouds interpretation; both of the above studies utilised a sedentary aging population, in contrast to the younger sedentary sample

that participated in the intervention in this study. It is possible that the participants' age or lifestyle had not yet induced aberrant epigenetic modifications that would cause a chronic inflammatory state, and thus, could explain the lack of the expected differential DNA methylation in the genes that were analysed.

While systemic concentrations of IL-6 and expression of the *IL6* gene have been relatively robustly shown to be manipulated by sedentary behaviour and reversed with regular exercise, no data thus far has been able to link this to epigenetic modifications in the form of differential DNA methylation. Since *IL6* methylation is closely linked with weight and inflammatory diseases, it seemed entirely logical that an intervention that reduced body mass and waist circumference, and therefore, likely subcutaneous fat, would elicit significant changes to the methylation of the gene concomitantly with modified transcription. As discussed in reference to *NFκB2* above, the CpG island under investigation by the primers that were designed specifically for this study may not have been modified, but as to whether other regions of the gene exhibited differential methylation cannot be elucidated at this time. It can be concluded, however, that exercise-induced changes to the fractional composition of the PBMC samples did not mask any true changes in DNA methylation over time given that flow cytometry data demonstrates that there were no significant changes in the relative proportion of any of the major cellular subsets.

Unfortunately, it was not possible to quantify whether changes between training and control groups occurred given that the methylated standards did not amplify on the control sample plates. Every effort was made to ensure that the experiments would function correctly; primers were designed in accordance with guidelines, which were then tested using gel electrophoresis to check for primer dimer formation and optimal annealing temperatures. There is a possibility that assay design was suboptimal, resulting in unsuccessful amplification of a large number of samples. It may have been beneficial to have conducted the experiments with various primer pairs designed for a similar region to increase the likelihood of adequate data, although constraints such as sample yield did not allow this approach. While msHRM has been shown to be a sensitive technique for measurement of DNA methylation, optimisation of each assay is evidently required which was unfortunately not possible within the scope of this project.

The primary focus of this study was to investigate the potential exercise-induced modifications to the DNA methylome following a progressive training intervention. Also of interest was the effect that training would have on the subsequent acute to response to a single bout of maximal exercise. Denham et al (2015[b]) reported training-induced changes to *miR21* and *miR210* CpG methylation concomitantly with altered expression of the genes, both at rest following the intervention, and acutely following a $\dot{V}O_{2max}$ test, suggesting that chronic epigenetic adaptations to exercise can indeed influence the subsequent transient response to exercise. However, they did not report whether DNA methylation was altered following the $\dot{V}O_{2max}$ test itself, only following the intervention. While it is likely that the intervention in the present study was a sufficient stimulus to

elicit longer term adaptations, the duration of the acute bout may not have been enough to elicit an acute change in DNA methylation within the genes and regions of interest. The studies in chapters 4 and 5 were able to show that a single bout of exercise is able to elicit significant alterations to the nuclear concentrations of *de novo* DNMTs, however, these bouts were much longer in duration, which could support this supposition.

An underlying aim throughout the entirety of this thesis has been whether manipulation of DNMT nuclear concentrations is due to cellular relocalisation, transcriptional changes, or both. The lack of change in relative *DNMT3A* expression following the training programme or acute bout suggests that previously observed modifications to nuclear concentrations of DNMT3A may not be due to transcriptional changes but rather cellular relocalisation of the protein, although it must be noted that two out of the three acute exercise bouts examined thus far within this thesis did not elicit changes to cellular concentrations of DNMT3A protein, and therefore may suggest that DNMT3A is not susceptible to exercise-induced modification. Unfortunately, the *DNMT3B* assay was not successful therefore, no data on *DNMT3B* expression is available to aid interpretation in this investigation. Post-transcriptional and translational modifications can lead to large discrepancies between mRNA and protein abundance (Vogel & Marcotte, 2013), however, the lack of DNMT3B primer amplification in this instance is likely due to poor assay design given that previous studies have successfully quantified the relative expression of the *DNMT3B* gene, rather than low expression of the gene. Future experiments should include measurements of PCR amplification efficiency in order to ensure accurate calculation of relative gene expression.

Even though an attempt was made to ensure that the sample size of the study was sufficient, a lack of data within this sub-discipline and inconsistencies in findings due to a vast number of methodological differences between studies meant it was difficult to ensure statistical power, and thus, it cannot be ruled out that the inconclusive findings may be due to sample size.

Despite a lack of significant effects of exercise-conditioned plasma on DNA methylation, *in vitro* stimulation with cytokines alone elicited a vastly different response. The high dose of IL-6 (10 $pg \cdot ml^{-1}$), similar to typical post-endurance exercise plasma concentrations, caused significant promoter hypomethylation of the *IL6* gene compared with a lower dose of 1 $pg \cdot ml^{-1}$. Previous data has shown that IL-6 can cause promoter hypermethylation; however, gene and cell line specificity mean that this does not directly contradict the *IL6* hypomethylation observed in this investigation. Given the previously reported significant elevation in DNMT3A and DNMT3B nuclear concentrations following stimulation with IL-6 (see chapter 3), it was surprising to observe promoter hypomethylation, and not vice versa. DNMT-independent mechanisms must have caused this reduction therefore, and since protein concentrations or expression of the active demethylase *TET* were not included in analyses, it is difficult to elucidate how this hypomethylation occurred.

In contrast, the same concentrations of IL-1β caused significant an elevation in *IL6* promoter methylation, which appears counterintuitive; promoter methylation is closely correlated with transcriptional silencing, however, IL-1β is widely regarded to induce IL-6 production in a number of cell types including monocytes (Tosato & Jones, 1990). Mechanistically, IL-1β has been shown to up- and down-regulate both *DNMT3A* and *DNMT1* expression in lung fibroblasts and RA fibroblast-synoviocytes, respectively. In this instance, it would be logical to assume that nuclear concentrations, activity, or transcription of the *de novo* DNMTs were elevated, resulting in the observed hypermethylation which is consistent with the findings presented in chapter 3. Functionally, when combined with other results presented in this thesis, these data support the supposition that inflammatory cytokines are significant regulators of both DNMTs, and at certain loci, modifications to DNA methylation itself.

Unexpectedly, TNFα did not elicit changes in DNA methylation of any of the genes, despite a body of experimental data demonstrating that TNFα is able to alter expression of all three functional DNMTs and *SOCS3* promoter methylation, although as previously alluded to, considerable cell specificity exists, potentially accounting for the lack of significant effects.

Of note is that each experimental condition was only performed in duplicate and analysed in triplicate, therefore this low number and relatively large variation could explain the lack of statistical significance with regard to *NFκB2* methylation following stimulation with IL-6 or IL-1β, although there does appear to be a visual elevation and reduction, respectively. Furthermore, msHRM has only been shown to be sensitive to 1% methylation, whereas a lot of samples were below this threshold, and therefore, these data would need to be interpreted with caution even in the event of a statistically significant outcome.

7.4.1 Conclusion

Overall, it is difficult to make any firm conclusions based on the data presented within this chapter. *In vitro* cytokine stimulation data, do however, corroborate results outlined in section 1.5, and support the posit that inflammatory cytokines are able to significantly alter DNA methylation in PBMCs, plausibly *via* cellular relocalisation of DNMTs. As to whether TET-dependent active demethylation or passive demethylation due to cytoplasmic transport of DNMTs accounted for the IL-6 induced hypomethylation of the *IL6* gene has not been elucidated.

Quantification of the relative expression of the genes would have yielded important information as to whether methylation changes did indeed result in functional changes to mRNA or protein concentrations. Furthermore, Human Methylation 450k or EPIC beadchips, which analyse over 485,000 and 850,000 methylation sites in the genome, respectively, would allow characterisation of the greater picture in terms of exercise-induced methylation changes.

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

8 General Discussion, Conclusions and Future Directions

8.1 General Discussion

8.1.1 Overview

Much research focus is given to genetic polymorphisms associated with sporting performance and disease susceptibility, however, epigenetic mechanisms which possess an essential role in transcriptional regulation are only starting to be acknowledged within exercise physiology research. As such, the study of epigenetics is still relatively contemporary, but is certainly a worthwhile area for investigation in order to fully understand how exercise exerts a multitude of changes at a transcriptional level.

The research presented within this thesis attempted to elucidate the underlying molecular mechanisms that govern exercise-induced epigenetic modifications. Existing data suggest that a number of proteins, in particular inflammatory cytokines, are able to regulate the expression and cellular localisation of DNMTs. Based on this, the objective was to characterise acute changes that occur to the *de novo* DNMTs following a single bout of exercise, and the involvement of exercise-induced systemic inflammation in this process. How changes in nuclear concentrations of these methylation-regulatory enzymes then translate into functional changes to the DNA methylome was also of interest. Furthermore, regular exercise appears to reduce inactivity-associated systemic inflammation, suggesting a cyclic relationship between exercise, inflammation, and epigenetic modification. Based upon this, the final overall objective was to elucidate whether regular exercise would exert significant modifications to promoter or first exon methylation of a number of genes associated with chronic low-grade inflammation in a sample of previously sedentary participants.

The pilot investigation presented in chapter 3 corroborated previous *in vitro* data that recombinant IL-6 is able to manipulate nuclear concentrations of DNMT3A and DNMT3B in human PBMCs. PBMCs that were cultured with plasma isolated from exercising participants before and after a number of acute exercise bouts exerted contrasting effects on cellular concentrations of the *de novo* DNMTs; following 120 minutes of treadmill running interspersed with short sprint periods, nuclear concentrations of DNMT3B were significantly attenuated concomitantly with an elevation in plasma IL-6 concentrations, whereas in contrast, circulating changes following a 24 hour ultra-endurance event elicited a significant augmentation of DNMT3A, in addition to plasma concentrations of IL-10, IL-6, IL-8, and TNFα. Despite the stark contrast in results, both studies strongly support the supposition that large, transient elevations in inflammatory cytokines are important regulators of exercise-induced epigenetic modifications. In support of this, exercise-conditioned plasma following eccentric exercise which did not elicit a transient elevation in inflammatory cytokines resulted in no significant alterations to nuclear or cytoplasmic DNMT

concentrations, or global DNA methylation. It is also of interest that the data presented in chapters 3 to 6 consistently shows that DNMT3A is less abundant within the nuclei of PBMCs, possibly suggesting that DNMT3B may be a more important regulator of DNA methylation in immune cells. Furthermore, as previously alluded to, this relationship between inflammation and epigenetic modification is likely to be bidirectional, and with that in mind, methylation within the *IL6* promoter, and *NFκB2* and *ASC* first exon regions was investigated following a seven week exercise intervention. No significant methylation changes were observed following the training programme, or following the subsequent acute maximal bout. Inflammatory cytokines did elicit significant alterations to the methylation of the *IL6* promoter region however.

8.1.2 Influence of Exercise-conditioned Plasma on DNMTs

The large decrement in DNMT3B and elevation in DNMT3A nuclear concentrations following stimulation with exercise-conditioned plasma could have been due to cellular relocalisation or transcriptional up- or downregulation, either alone or in combination. IL-6 has previously been shown to cause nuclear translocation of DNMT1 (Hodge et al., 2007) and high concentrations of glucose can cause cytoplasmic translocation of DNMT3A (Zhang, Li, Zhang, Huang, Wang, & Yi, 2016), while a number of inflammatory mediators and even muscular contraction can alter *DNMT* transcription.

The contrasting effect of exercise-conditioned plasma on nuclear concentrations of DNMT3A and DNMT3B following the two endurance exercise bouts is difficult to explain. Post-exercise IL-6 reached similar concentrations in both studies, and therefore, the differential response is unlikely to be due this cytokine alone. Plasma concentrations of other inflammatory cytokines were not quantified following the 120 minute protocol, but TNFα, IL-8, and the anti-inflammatory IL-10 were found to be significantly elevated following the ultra-marathon. Given that 100 $ng{\cdot}ml^{-1}$ of TNFα and IGF-1 in combination caused an increase in *DNMT1* and *DNMT3B* mRNA compared with each cytokine alone, and *DNMT3A* compared with controls, albeit using coronary artery cells (Dhar et al., 2013), it could be hypothesized that a combination of exercise-induced systemically elevated cytokines causes differential DNMT regulation in contrast to each individual cytokine alone. It is also plausible that although plasma IL-6 was augmented following the 120 minute protocol, the anti-inflammatory mediator IL-10 may not have been significantly elevated by the post-exercise sample point. In contrast, the ultra-marathon post-exercise sample was taken 24 hours after the initiation of exercise, and therefore the two-fold increase in IL-10 at this point likely indicates the beginning of inflammatory resolution. Given that different signalling cascades control pro- and anti-inflammatory pathways, at least with regard to IL-6 (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011), it is entirely possible that differential activation of these pathways due to the timeframe in which the samples were taken would be sufficient to elicit the contrasting downstream changes in nuclear DNMT concentrations.

It must be noted that multiple regression analysis revealed that the elevations in the inflammatory cytokines following the ultra-marathon were unable to predict the augmentation in nuclear DNMT3A, and thus, other circulatory factors likely contributed. Not only do circulating inflammatory profiles differ between endurance and eccentric exercise bouts, plasma miRNA concentrations have also been shown to be differentially regulated by acute eccentric (Banzet et al., 2013), resistance (Sawada et al., 2013), and aerobic exercise (Nielsen et al., 2014). While a number of miRNAs have been shown to target and alter the transcription of all three *DNMT*s (section 1.2.2), it is currently unclear as to whether miRNAs located in the plasma possess similar regulatory roles. Contrasting plasma miRNA profiles have been reported in endurance and strength trained athletes (Wardle et al., 2015), which also highlights the potential for different exercise modalities to elicit contrasting epigenetic responses to an acute bout.

In vitro stimulation of mesangial cells with high concentrations of glucose causes cytoplasmic translocalisation of DNMT3A via ERK/MEK signalling without altering *DNMT3A* expression, consequently causing increased *CTGF* expression (Zhang et al., 2016). Given that hyperglycaemia can elicit an elevation in inflammatory gene expression in various cell types (Wasinksi et al., 2014; Witkowski, Guhanarayan, & Burgess, 2016), the IL-6/ERK/MEK pathway could explain Zhang et al's finding. In the context of exercise, Marliss and Vranic (2002) reported that cycling at 100% of $\dot{V}O_{2max}$ caused a significant augmentation of plasma glucose. Conversely, Coyle, Coggan, Hemmert, and Ivy (1986) showed that cycling to fatigue at 70% of $\dot{V}O_{2max}$ was preceded by a decline in both plasma glucose and muscle glycogen, which was only delayed for an hour when fed additional carbohydrates. Thus, it was concluded that intense exercise can result in elevations in plasma glucose due to a mismatch between glucose production and utilisation, but only at intensities over 80% of $\dot{V}O_{2max}$, whereas plasma glucose concentrations tend to remain constant during moderate intensity exercise (Marliss & Vranic, 2002). It is likely, however, that the exercise bouts utilised throughout this thesis did not result in plasma hyperglycaemia; it is extremely probable that the 24 hour ultra-marathon resulted in a significant drop in muscle glycogen by the end of the race, thereby limiting glucose production. The drop jump protocol utilised in chapter 6 did not involve a large metabolic demand, and therefore, it is plausible that plasma glucose remained constant. Furthermore, the protocol utilised in chapter 4 was previously shown to not alter plasma glucose immediately following exercise (Walshe et al., 2011). As such, within the context of the data presented in this thesis, perturbations in plasma glucose concentrations would not appear to explain the exercise-conditioned plasma induced alterations to the nuclear concentrations of DNMTs.

It is well documented that epigenetic alterations do not occur in isolation, and that DNA methylation and histone modifications are closely linked. Another mechanism that could explain the documented effects of exercise on DNA methylation concerns the overlap between these two modifications, as DNMTs have been known to bind and correlate with HDACs (Fuks et al., 2000;

Gu et al., 2013; Rountree et al., 2000); attenuated nuclear HDAC concentrations following 60 minutes of cycling (McGee, Fairlie, Garnham, & Hargreaves, 2009) could have been due to enhanced nuclear export via CaMK signalling (McKinsey, Zhang, Lu, & Olson, 2000). Since muscular contractions can augment CaMKII activity (Rose, Kiens, & Richter, 2006) and induce DNA hypomethylation in skeletal muscle (Barrès et al., 2012; Lucas et al., 2012), cytoplasmic relocalisation of HDACs and bound DNMTs may be a mechanism by which exercise causes attenuated nuclear concentrations of these epigenetic enzymes and subsequent DNA hypomethylation. However, this pathway has been documented in skeletal muscle and not circulating immune cells.

While the data presented in chapters 3, 4, and 5 differs dramatically, all three studies support the hypothesis that exercise-induced changes to circulating inflammatory cytokines are able to cause significant changes to the nuclear concentrations of the *de novo* DNMTs. However, since no changes in nuclear or cytoplasmic concentrations of either DNMT occurred following the damaging eccentric protocol, it was not possible to elucidate whether they exhibit a differential time course of modification as hypothesized.

These data alone were unable to clarify whether changes in nuclear concentrations were due to cellular relocalisation or transcriptional changes. As previously alluded to, alterations in cellular concentrations of DNMT3A and DNMT3B could also be due to transcriptional up- or downregulation; a 50% attenuation of skeletal muscle *DNMT3B* mRNA has been reported following three hours of cycling at 60% of v$\dot{V}O_{2max}$ (Laye & Pedersen, 2010). The Ca^{2+} ionophore ionomycin elicited an approximate 40% reduction in both *DNMT3A* and *DNMT3B* mRNA in differentiated myotubes. Results presented in chapter 7 showed that expression of *DNMT3A* was unaltered following stimulation with exercise-conditioned plasma following a progressive exercise intervention, which appears to contradict the aforementioned studies. Relative *DNMT3B* expression data from this investigation is not available, and thus, inferences must be made cautiously.

Despite this, *in vitro* data suggests that transcriptional changes due to inflammatory proteins are highly likely. Exercise-induced elevations in PGE_2 concentrations have been reported following a marathon (Demers et al., 1981) and run to exhaustion at 80% of $\dot{V}O_{2max}$ (Venkatraman et al., 2001). Given the duration and intensity of the two endurance exercise bouts, increased plasma PGE_2 concentrations were likely to have occurred. When combined with the *in vitro* data discussed in section 1.5.3, manipulations in circulating PGE_2 could also have elicited changes to *DNMT* expression. IL-6, TNF-α, IL-1β, IGF-1 also exert significant alterations to the transcription of *DNMT1, DNMT3A,* and *DNMT3B* in a variety of cell types (section 1.5), further substantiating this supposition.

8.1.3 Influence of Exercise-conditioned Plasma on DNA Methylation

As expected, due to a lack of change in nuclear DNMT concentration, no significant alterations in the amount of global DNA methylation occurred following the damaging eccentric protocol. This corroborates previous data that global methylation remained unchanged in PBMCs following the same protocol (Robson-Ansley et al., 2014), and in NK cells following a marathon (Zimmer et al., 2015). Global DNA methylation may be a useful measure in epidemiological studies, however the effects of an acute stimulus such as exercise appears to require more sensitive measures. Moreover, it is difficult to isolate the functional downstream consequences of altered global methylation, given that hypermethylation, for example, within different regions of the same gene can promote such contrasting effects.

Evidence suggests that regular exercise can favourably alter the methylation profile of genes involved in inflammatory pathways such as *NFκB* (Zhang et al., 2015) and *ASC* (Nakajima et al., 2015), while Denham et al. (2015[b]) have shown that four weeks of training, albeit at a higher intensity, elicited a number of gene-specific methylation alterations in leukocytes. Conversely, Duggan et al. (2014) did not report any significant effects of 12 months of training on leukocyte methylation, however global methylation was the outcome measure, the weaknesses of which have been discussed already within this thesis. Results reported herein were unable to corroborate these previous findings. As discussed in chapter 7, despite the positive physiological effects of the training in terms of improved cardiovascular fitness and body composition, methodological limitations such as small sample size and possible suboptimal assay design may have masked the true gene-specific modifications to the methylome, and thus, it would be unwise to conclude that regular exercise simply does not cause any changes to methylation within these genes.

8.1.4 Influence of Inflammatory Cytokines on DNMTs and DNA Methylation

Physiological and supra-physiological concentrations of rIL-6 were shown to significant augment nuclear concentrations of both DNMT3A and DNMT3B in chapter 3. An abundance of *in vitro* research has previously investigated the effect IL-6 on DNMTs, and the associated pathways by which these proteins interact. IL-6 may augment *DNMT1* expression via JAK2/STAT3 signalling (Huang et al., 2016; Liu et al., 2015[a];Zhang et al., 2006). Additionally, STAT3 has been shown to bind to DNMT1 and causes epigenetic silencing by targeting DNMT1 to promoters (Lee et al., 2012; Zhang, Wang, Marzec, Raghunath, Nagasawa, & Waski, 2005). The PI3K/AKT signalling pathway also appears to regulate at least DNMT1 in a variety of cell lines; Sun et al. (2007) reported a positive correlation between DNMT1 protein levels and AKT phosphorylation in a large number of human cell lines and bladder cancer tissues. Inhibition of AKT resulted in DNA hypomethylation, presumably due to a reduction in DNMT1 protein stability or abundance, given that AKT enhanced DNMT1 protein stability and elevated protein concentrations independently from changes in mRNA.

While much of the literature utilises supra-physiological concentrations of IL-6 and disease-specific cell lines, the data presented in this thesis is novel in that normal physiological ranges of rIL-6 were used to stimulate healthy human PBMCs. Furthermore, a lot of these previous studies focus specifically on the effects of the 'maintenance' DNMT, DNMT1, with little evidence reported thus far linking IL-6 with the *de novo* DNMTs. In hepatocellular carcinoma cell lines however, PI3K signalling regulates *DNMT3B* expression (Mei et al., 2010), whereas in prostate cancer cells, PI3K/AKT inactivation attenuates both *DNMT1* and *DNMT3B* expression (Agarwal et al., 2013). Nuclear DNMT concentrations can also be augmented via cellular relocalisation; PI3K/AKT signalling mediates the phosphorylation of DNMT1's nuclear localisation signal, thereby causing translocation into the nucleus from the cytoplasm (Hodge et al., 2007).

The data presented in chapter 3 is difficult to interpret in isolation, but provides support to the hypothesis that IL-6 does indeed cause elevations in nuclear concentrations of DNMT3A and DNMT3B in PBMCs, either by transcriptional upregulation or cellular translocation. The obvious concern with making comparisons between the data presented herein and the literature is that physiological responses differ dramatically between healthy and diseased cells, and much of the data describes mechanistic links between IL-6 and DNMT1, not DNMT3A or DNMT3B. Thus, in isolation, any conclusions regarding the signalling pathways by which rIL-6 is able to exert significant influence of the nuclear concentrations of DNMT3A and DNMT3B, must be made with caution. These results do, however, support the exercise-conditioned plasma data, and the original supposition that IL-6 is clearly a mediator of epigenetic-regulatory machinery.

In addition to the ability to elicit significant changes to nuclear concentrations of these enzymes, inflammatory cytokines were shown to exert downstream functional effects in the form of modified DNA methylation. While IL-6 is able to modify DNMTs *in vitro*, as previously discussed, in addition to promoter methylation of tumour suppressor genes, IL-1β has only thus far been shown to affect *DNMT1* and *DNMT3A* expression. It is unsurprising that these cytokines would result in altered DNA methylation as reported in chapter 7, although it did appear paradoxical that IL-1β induced *IL6* promoter hypermethylation, as this modification is typically associated with transcriptional silencing whereas IL-1β has robustly been shown to experimentally induce IL-6 production in immune cells. This highlights the complexity of epigenetic modifications in differing genomic regions, and supports the necessity to quantify concomitant protein or mRNA levels in addition to methylation changes across a number of CpG islands within a gene's upstream regulatory regions such as the transcription start site.

With all of the above discussion points in mind, a complex cascade of events linking exercise-induced circulatory changes and epigenetic modification clearly exists. Figure 8.1 represents a hypothetical pathway, based upon data presented and discussed thus far within the entire thesis, by which these transient systemic changes alter nuclear protein concentrations of DNMTs leading to epigenetic modifications.

8.1.5 Strengths and Limitations

As previously discussed, the use of a standardised cell sample in all experimental conditions means that interpretation of these data is difficult in terms of generalisation to a wider population. However, in order to fully elucidate the role of exercise-induced circulatory changes on subsequent alterations in cellular concentrations of epigenetic markers, it was essential that individual differences in polymorphisms which may alter susceptibility to epigenetic change were controlled for. Therefore, within the scope of this line of investigation, it was deemed an appropriate experimental decision to make. Furthermore, PBMCs represent a more homogenous cell population than whole blood or peripheral blood leukocytes which are often utilised, yet still maintain the advantage of being a relatively non-invasive and inexpensive sample for investigation.

A strength of the series of studies presented herein is that a wide range of exercise bouts were utilised, each with their own unique physiological demand, thus allowing a more complete characterisation of exercise-induced epigenetic changes. Further quantification of the vast number of exercise-induced changes to circulatory proteins and molecules would have greatly aided in the interpretation of data.

A hypothesis driven targeted approach to DNA methylation investigation meant that a strong rationale was developed for the genes that were selected to be investigated. Nonetheless, the use of a complex contemporary technique such as msHRM resulted in a large number of missing datapoints; the lack of data for the control group from chapter 7 meant that it was not possible to fully interpret the effects of a training intervention on gene-specific methylation. Although extremely costly, a methylation sequencing approach would have allowed more complete characterisation of the genome-wide effects of exercise on the DNA methylome. Had changes in DNA methylation occurred following stimulation with exercise-conditioned plasma, concomitant quantification of gene expression would also have been beneficial.

Measurement of protein concentrations of DNMTs within the cellular compartments, and not protein activity, could also be deemed to be a limitation. The decision to quantify abundance was based primarily on *in vitro* data that shows that rIL-6 is able to elicit nuclear translocation of DNMTs, and the subsequent hypothesis that exercise-induced elevations in plasma IL-6 would produce a similar alteration in cellular DNMT concentrations. In order to compare data between studies it was deemed necessary to continue utilising the same approach.

Finally, the signalling cascades by which inflammatory cytokines elicit modifications to the methylome could be clarified using targeted RNA sequencing technologies, such as the NFκB panel offered by Illumina. Thus far, it has been difficult to hypothesize the pathways by which IL-6, for example, causes nuclear translocation of DNMTs and subsequent methylation changes, as such a vast array of cell types are utilised within the available literature.

8.2 Conclusions and Future Directions

The data presented in this thesis builds upon the study conducted by Robson-Ansley et al. (2014) and furthers the understanding of the mechanisms underlying exercise-induced epigenetic modifications. The data consistently demonstrate that exercise-induced changes to circulating inflammatory cytokines exert significant influence over the nuclear localisation of the *de novo* DNMTs, DNMT3A and DNMT3B, and is the first data to demonstrate the molecular mechanisms by which exercise can elicit modifications to the methylome in PBMCs. Furthermore, despite inconclusive results on the effects of exercise-conditioned plasma on *DNMT* expression and DNA methylation itself following exercise training, previous literature suggests that exercise can indeed modify inflammatory gene methylation, albeit in different populations. Firm conclusions within this context are therefore confounded by a number of methodological issues.

This is preliminary research into the mechanisms that underpin the complex relationship between physical activity and inflammation. Promoter or first exon methylation of select genes involved in key inflammatory pathways may in future become a useful biomarker by which to measure the effectiveness of exercise interventions on chronic inflammation and disease susceptibility. The postulation that over the long term, moderate amounts of exercise may indeed be more health-beneficial than intense and excessive training volumes (Radak, Chung, Koltai, Taylor, & Goto, 2008) could be tested by measuring inflammatory gene promoter methylation and protein levels longitudinally following varying exercise programmes.

Due to the paucity of literature within the 'exercise epigenomics' domain, a vast number of potential research avenues remain. Given the complex nature and overlap of different epigenetic modifications, when combined with genetic factors such as polymorphisms which may alter susceptibility to epigenetic change, collaborative approaches appear necessary in order to fully elucidate the mechanisms by which exercise alters phenotypes.

With a focus on the scope of the research presented in this thesis, it would be beneficial to further characterise the role of exercise-induced circulatory changes on epigenetic modification, and the underlying pathways which mediate changes. Specifically, the inclusion of other inflammatory measures, in particular TNFα, IL-1β, and PGE_{2}, based on *in vitro* data, may aid in the interpretation of how transient inflammation may elicit changes to the methylome. Furthermore, miRNAs are well known to interact with epigenetic-regulatory enzymes, and recent evidence suggests that exercise can alter the circulatory miRNA profile. Exercise-induced changes to intra- or extracellular Ca^{2+} may significantly contribute to inflammatory gene upregulation within immune cells, and thus, warrants further investigation also.

In addition to the above recommendations, more *in vitro* studies, using techniques such as chromatin immunoprecipitation, may allow elucidation of the interaction between proteins in the complex cascade of events that mediate epigenetic modifications, which in turn may help to

explain the precise pathways involved. The inclusion of TET in these analyses would also aid the understanding of the methylation/demethylation process which is both dynamic and complex.

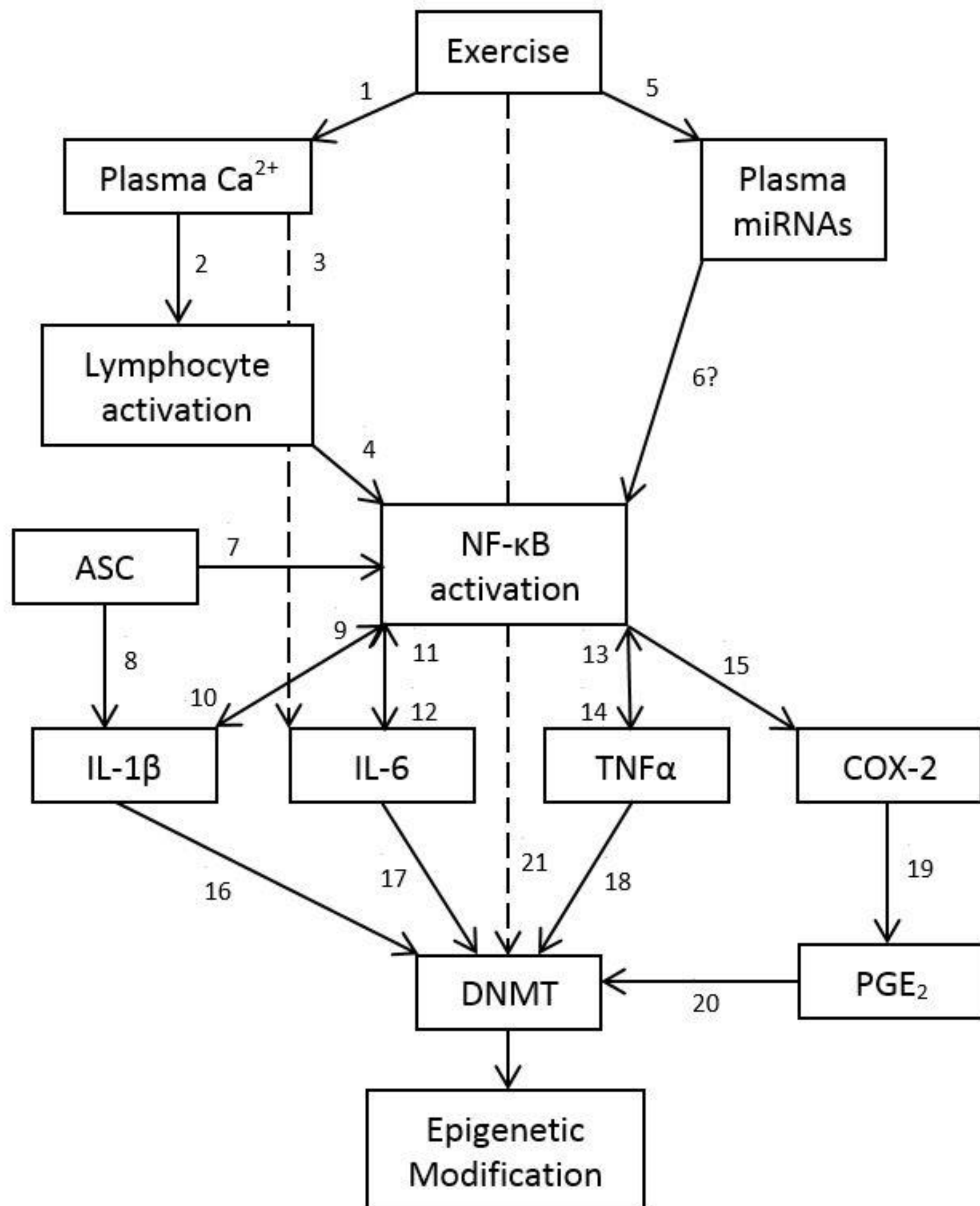

Figure 8.1 *- Schematic representing a hypothetical pathway, based on data from a variety of cell types, by which exercise may cause circulatory changes that elicit epigenetic modifications (Numbers indicate references which can be found in full in appendix L).*

CHAPTER 9

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CHAPTER 10

APPENDICES

Appendix A – Summary of literature in section 1.5.

Study	Cell Line/Tissue	Cytokine/ Stimulus (concentration)	Epigenetic Modification/Enzyme	Notes
IL-6				
Hodge et al. (2001)	K562 (leukaemia) cell line	IL-6 (100 ng·ml^{-1})	↑ *DNMT1* expression	
Hodge et al. (2005)	KAS 6/1 (multiple myeloma) cell line	IL-6 (10 ng·ml^{-1})	↑ *DNMT1* expression	IL-6 maintains PM of *p53*
Wehbe et al. (2006)	Mz-ChA-1 (cholangiocarcinoma) cell line	Cells transfected with IL-6 = IL-6 overexpressing cell line, Mz-IL-6		PM not altered for most genes; PM↑ *CASP8, Survivin, HoxA2*; PM↓ *TFF1, Tastin*
Zhang et al. (2006)	PB-1, 2A, 2B (T cell lymphoma) cell lines			STAT3 (activated by IL-6) induces *DNMT1* expression
Hodge et al. (2007)	HEK293 (embryonic kidney) cell line	IL-6 (100 ng·ml^{-1})	DNMT1 nuclear translocation	Phosphorylation of nuclear localisation signal by AKT
Braconi et al. (2010)	KMCH-1, Mz-ChA-1, TFK-1 (human cholangiocarcinoma), and H69 (human non-malignant cholangiocyte) cell lines	Cells transfected with IL-6 = IL-6 overexpressing cell lines, KM-IL-6 and Mz-IL-6	↑ *DNMT1* expression in Mz-IL-6 and KM-IL-6 cells compared with controls	IL-6 regulated activity of DNMT1 by modulation of miR-148a and miR-152 ↓ expression of tumour suppressor genes *Rassf1a*, *p16INK4a*, *MGMT*
Foran et al. (2010)	IBD-CRC tumours		↑ *DNMT1* expression compared with sporadic CRC tumours	*DNMT3B* overexpression in CRC tumours
	HT29, HCT15, HCT116 (colon cancer) cell lines	IL-6 (100 ng·ml^{-1})	↑ *DNMT1* expression	↑ PM of tumour suppressor genes

Li et al. (2012)	CRC patient colon biopsies		IL-6 associated with ↑ *DNMT1* expression	IL-6 induced *DNMT1* expression correlated with *SOCS3* PM, resulting in ↑ STAT3 signalling
Liu et al. (2015[a])	A549 (lung cancer) cell line	IL-6 (10 ng·ml^{-1})	↑ *DNMT1* expression; no change in *DNMT3A/3B*	IL-6/JAK2/STAT3 pathway upregulates *DNMT1*
Huang et al. (2016)	Pancreatic cancer cells	IL-6 (100 ng·ml^{-1})	↑ *DNMT1* activity	IL-6/STAT3 promotes *SOCS3* PM via *DNMT1* expression and recruitment to promoter region
Other Cytokines				
Barrès et al. (2009)	Human myotube cells	TNFα (1 µM)	↑ non-CpG methylation	*PGC1α* promoter
Acharyya et al. (2010)	Mouse myoblast cells	TNFα (unspecified concentration)	DNMT3B recruitment	Inhibition of Notch-1 (transmembrane protein), attributed to Ezh2 (histone methyltransferase) and DNMT3B recruitment via NF-κB, resulting in hypermethylation
Huang et al. (2012)	IMR-90 (normal human lung fibroblast)	IL-1β (10 ng·ml^{-1})	↑ *DNMT3A* expression ↑ *DNMT1* expression	Effects of IL-1β blocked by aspirin
		Aspirin (200 µM)	↓ *DNMT3A* expression	
Nakano et al. (2013)	Fibroblast-like synoviocytes (RA patients)	IL-1β (2 ng·ml^{-1})	↓ *DNMT3A* expression ↓ *DNMT1* expression	
		TNFα (50 ng·ml^{-1})	↓ *DNMT3A* expression	
Dhar et al. (2013)	Human coronary artery smooth muscle cells	TNFα (100 ng·ml^{-1})		↑ *SOCS3* expression at 24 hours TNFα activated NK-κB

		IGF-1 (100 ng·ml^{-1})		↑ *SOCS3* expression at 24 hours IGF-1 phosphorylated STAT3
		TNFα + IGF-1	↑ *DNMT1, DNMT3A,* and *DNMT3B* expression vs control Knockdown of *DNMT1* = ↑ *SOCS3* expression	↑ *SOCS3* PM ↓ *SOCS3* expression at 12 hours
Prostaglandin E_2				
Huang et al. (2012)	Fibroblasts (fetal and adult)	PGE_2 (10^{-6} to 10^{-9} M i.e. 1 μM to 1nM)	↑ *DNMT3A* expression/activity	GM ↑ at 48h (not 24h) ↑ expression and activity of Sp1 and Sp3 transcription factors
	RAW macrophages	PGE_2 (10^{-6} to 10^{-9} M i.e. 1 μM to 1nM)	↓ *DNMT3A* expression ↓ *DNMT1* expression	
Xia et al. (2012)	CRC specimens	PGE_2	Positively correlated with *DNMT1* and *DNMT3B*	
	3 human CRC cell lines (unspecified)	PGE_2 (unspecified concentration)	Direct upregulation of *DNMT1* and *DNMT3B*	
	LS-174T (human colorectal carcinoma) cell line	PGE_2 (unspecified concentration)		↑ PM and ↓ expression of *CNR1, MGMT, CDKN2B, MLH1*
	Apc$^{Min/+}$ mice	PGE_2 (unspecified concentration)	↑ *DNMT1* and *DNMT3B* expression in colonic tumour epithelial samples.	↑ PM and ↓ expression of *CNR1, MGMT, CDKN2B, MLH1*

Caffeine/Ionomycin (Ca^{2+})				
Laye & Pedersen (2010)	*Vastus lateralis* skeletal muscle	3 hours cycle @60% $\dot{V}O_{2max}$	50% ↓ *DNMT3B* mRNA	
	Proliferating myoblasts	Ionomycin (0.1 µM)	No change in *DNMT3A* or *DNMT3B* mRNA	
	Differentiated myotubes	Ionomycin (0.1 µM)	↓ *DNMT3A* and *DNMT3B* mRNA (~40%)	
Barrès et al. (2012)	L6 myotubes (rat skeletal muscle)	Caffeine (5mM)		↓ PM of *PGC1α, TFAM, MEF2A, CS, PDK4;* ↑ mRNA of *PGC1α, TFAM, MEF2A, CS, PDK4*
		H_2O_2 (1mM)		↑ PM of *TFAM, MEF2A, CS*
		Ionomycin (1 µM)		↑ mRNA of *PGC1α, TFAM, MEF2A;* No change in PM
Anti-Oxidants				

Blaschke et al. (2013)	Mouse embryonic stem cells	Vitamin C (100 µg·ml^{-1})	↑ TET activity TET1 binding is enriched near TSS of genes affected by treatment No change in *TET* and *DNMT* gene expression Glutathione does not change TET1 activity	Global ↑ in 5-hmC (lost after 3 days of withdrawal) Most methylated promoters gain 5-hmC at 12hrs and return to baseline by 72hrs 5-mC is progressively lost at 12 and 72hrs (2-fold ↓ PM at 61% of analysed promoters by 72hrs) 5-hmC gain and 5-mC loss occur at same genomic locations near TSS Attenuated increase in 5-hmC in *TET1* knockout cells Vitamin C treatment does not affect 5-mC or 5-hmC in *TET1/2* double knockout cells
Yin et al. (2013)	TET-transfected HEK-293T (human embryonic kidney) cell line	Ascorbic acid (various concentrations)	↑ TET1 catalytic domain activity	5-hmC ↑ 4 (50 µM) and 7 (500 µM) fold 5-fC ↑ 4.6 (50 µM) and 8.9 (500 µM) fold ↑ in 5-hmC and 5-fC accompanied by ↓ in 5-mC

	Mouse embryonic stem cells			5-hmC ↑ 3.7 fold (100 μM over 24hrs) 5-fC ↑ 10.6 fold 5-caC ↑ 20 fold 5-mC ↓ 13% after 1 day; 32-40% after 2-3 days No sig. change in *TET1* expression, therefore AA influences the catalysis of TET 5-hmC, 5-fC, and 5-caC barely detectable in *TET1/2* double knockout cells ↓ in 5-mC replicated *in vivo* (mouse liver/cerebrum)
Minor et al. (2013)	Mouse embryonic fibroblasts	Ascorbate (various concentrations)	TET	Dose-dependent ↑ in 5-hmC (24hrs; 0-1000 μM) No change in TET expression (5-hmC still enhanced in TET-deficient cells)

	HEK-293T (human embryonic kidney cells – transformed with large T antigen)/HeLa cell line	Ascorbate (various concentrations)	↑ 5-hmC (10 μM)
Contraction			
Lucas et al. (2012)	Mouse soleus muscle	*Ex vivo* contraction	↓ PM of *PPARδ, PDK4, CS, PGC1α* (at 30mins and 270mins post-contraction) ↑ mRNA *PPARδ, PDK4, CS, PGC1α* (at 180mins)
Barrès et al. (2012)	Mouse soleus muscle	*Ex vivo* contraction	↓ PM of *PGC1α, PPARδ, PDK4, MYOD1, MEF2A* @45 mins post-contraction ↑ mRNA *PGC1α, PPARδ, PDK4* @180 mins post-contraction

Appendix B – Summary of literature in section 1.6.

Study	Population	Activity	Measurement/Tissue	Results
Habitual PA – Global Methylation				
Zhang et al. (2011)	131 men/women, >45 years, various ethnicities, no history of heart/kidney disease or cancer	PA assessed over 4 days using accelerometry	GM (LINE-1) in peripheral blood	>30 minutes of PA per day = ↑ LINE-1 methylation compared with those who performed <10 minutes per day
Gomes et al. (2012)	126 men and women. Elderly (aged 60-88)	PA assessed by steps per day over 1 week	GM (Imprint Methylated DNA Quantification Kit) of peripheral blood leukocytes	No sig. correlation between PA and GM
Luttropp et al. (2013)	509 men/women, aged 70, healthy	Self-reported weekly PA participation assessed	GM (LUMA) in leukocytes	GM significantly correlated with activity level (after adjustment for gender, SBP, DBP, LDL and HDL cholesterol, serum triglycerides, smoking status, and BMI)
White et al. (2013)	647 women, aged 35-74, non-Hispanic, sister diagnosed with BC	PA (hours per week) retrospectively recalled for ages 5-12, 13-19 and previous 12 months	GM (LINE-1) in peripheral blood	PA levels above median for all 3 time periods = significantly ↑ GM vs. those below median
Habitual PA – Gene-Specific Methylation				
Coyle et al. (2007)	106 women without BC diagnosis, mean age = 43 years	Interviewer-administered lifetime PA questionnaire	PM of *APC* and *RASSF1A* genes in biopsied breast tissue using methylation-specific PCR	Lifetime, previous 5 years, and previous year PA inversely correlated with PM of *APC* but not *RASSF1A*
Slattery et al. (2007)	1154 colon cancer patients	Recall of activity level for previous year, and 10/20 years ago	Methylation-specific PCR of *MINT1*, *MINT2*, *MINT31*, *p16* and *hMLH1* following tumour biopsy	No sig. correlation between PA and selected markers

Yuasa et al. (2009)	106 male/female primary gastric carcinoma patients	Self-administered pre-cancer PA history questionnaire	Methylation-specific PCR of 6 tumour-related genes; *CDX2*, *BMP-2*, *p16*, *CACNA2D3*, *GATA-5*, *ER* following tumour biopsy	PA inversely correlated with *CACNA2D* methylation
Pirola et al. (2012)	45 non-alcoholic fatty liver patients	Questionnaire covering all forms of PA (hours per week)	Methylation-specific PCR of *MT-ND6*, *MT-CO1* and *D-loop* genes in liver mitochondrial DNA	Inverse correlation between PA and *MT-ND6* methylation
Ren et al. (2012)	237 female tai chi practitioners compared with 263 female beginners, aged 45-88	Experienced practitioners defined as >3 years, while beginners had just enrolled in beginner classes	Saliva DNA isolated from mouthwash, with methylation quantified at 60 CpG sites using Sequenom EpiTYPER platform	Differential methylation of 6 CpG sites in the experienced, compared with beginner, group
Zhang et al. (2012)	165 men/women, aged 18-78, college commuters	Block adult energy expenditure survey (assesses frequency and duration of 26 activities within the past year)	GM (LINE-1) and IL6 PM in leukocytes	No association between PA and LINE-1 methylation, or PA and *IL6* PM
Morabia et al. (2012)		Block adult energy expenditure survey (assesses frequency and duration of 26 activities within the past year)	GM (LINE-1) and IL6 PM in leukocytes	Public transportation users did not have lower levels of LINE-1 methylation or *IL6* PM vs car drivers
Lott et al. (2013)	85 men/women, aged 22-70, schizophrenia diagnosis	Questionnaire covering duration and intensity of PA over the week prior to the DNA sample being obtained	Pyrosequencing used to analyse 2 methylation sites of *COMT-s* promoter region in peripheral blood	Significant negative correlation between *COMT* promoter methylation and PA in Val/Val homozygous patients
Clarke-Harris et al. (2014)	40 mixed-sex children, measured at 5 and 14 years old	Measured annually (7 consecutive days) by accelerometry	*PGC1α* promoter CpG loci analysed by pyrosequencing of whole blood DNA	No interaction between PA and methylation at several *PGC1α* promoter CpG loci, however, methylation at 4 loci predicted adiposity independently

				from gene or PA
Shaw et al. (2014) [abstract]	253 elderly white males, 137 elderly white females	Self-reported PA energy expenditure assessed over 8 years	Quantitative methylation-specific PCR of leukocytes	Hypermethylation of *TNF* in those who increased PA energy expenditure by 500 kcal or more per week. Hypomethylation of *IL-10* in those who increased vs. those who decreased by 500 kcal or more per week
Acute Exercise				
Laye & Pedersen (2010) [abstract]	8 healthy young men	3 hours of cycling at 60% $\dot{V}O_{2max}$	*Vastus lateralis* skeletal muscle biopsy and expression (PCR) of *DNMT3A* and *DNMT3B*	*DNMT3B* mRNA decreased by 50% immediately post-exercise
Barrès et al. (2012)	14 sedentary, healthy men/women	Cycle ergometer $\dot{V}O_{2peak}$ test	GM (MeDIP) of *vastus lateralis* skeletal muscle (PM of selected genes also quantified)	GM reduced following the acute exercise bout. PM decreased at *PGC1α*, *PDK4* and *PPARδ*. No change in PM of *MEF2A* or *MYoD1*
Robson-Ansley et al. (2014)	8 healthy, trained men	120 minutes of treadmill running at 60% v$\dot{V}O_{2max}$ followed by 5km time trial	HumanMethylation27 Beadchip analysis of PBMC samples	GM and CpG site-specific methylation remained unchanged. IL-6 protein levels correlated with CpG methylation at 11 CpG sites
Lane et al. (2015)	7 trained male cyclists	Evening session of HIIT(8x5min @82.5% PPO, 1min active recovery), followed by 120 mins (steady state @50% PPO) in the morning (having eaten either 8 g·kg^{-1} BM of CHO before the session and slept with without eating, or 4 g·kg^{-1} BM of CHO before the session and 4 g·kg^{-1}	*Vastus lateralis* skeletal muscle biopsy Bisulfite converted DNA amplified by PCR then pyrosequenced (Qiagen Q24 Advanced Pyromark	Following 120min morning session, PM ↑ 4h post-120min in *COX4I1* (vs. post-120min/fasted group only) and *FABP3* (vs pre-120min/both groups) Only *FABP3* mRNA ↑ post-120min vs

		BM of CHO before sleeping)	System)	rest PM of *PPARδ* increased only in fasted group vs baseline mRNA ↑ 4h post-120min vs baseline and post-120min in fed group only
Zimmer et al. (2015)	14 cancer survivors (male and female; breast/colon/prostate) and 14 controls	Half-marathon	Global methylation of NK cells using primary monoclonal antibodies	No change
Exercise Interventions				
Alibegovic et al. (2010)	10 young, healthy men	9 days bed rest followed 4 weeks of retraining (cycle ergometry for 30 mins per day, 6 days per week at 70% $\dot{V}O_{2max}$)	Sequencing PCR of *vastus lateralis* skeletal muscle	Sig. hypermethylation of 1 (of 3) CpG site in *PGC1α* promoter following 9 days bed rest *PGC1α* methylation not fully restored to baseline following 4 weeks retraining *PGC1α* mRNA ↓ following bed rest, which did not return to baseline following retraining
Nakajima et al. (2010)	162 controls (aged 40-87), 274 exercise group (aged 41-86), 37 young controls (aged 18-22), healthy, Japanese	6 months of several sets of 3 minute low-intensity walking at 40% of $\dot{V}O_{2peak}$, followed by 3 minutes of high intensity walking above 70% $\dot{V}O_{2peak}$, at least 2 days per week	Peripheral blood *ASC* gene methylation (pyrosequencing)	*ASC* methylation decreased with age, while the exercise intervention attenuated this age related decrease

		Tracked by accelerometry		
Zhang et al. (2015)	6 control, 7 exercise	6 months of several sets of 3 minute low-intensity walking at 40% of $\dot{V}O_{2peak}$, followed by 3 minutes of high intensity walking above 70% $\dot{V}O_{2peak}$, at least 2 days per week	Genome-wide microarray assay (Agilent Human CpG Island Array 244K) of peripheral blood	Over 40 differentially methylated genes after training, including *NFκB2*
	20 exercise subjects	Tracked by accelerometry	Pyrosequencing (Pyromark Q24ID) of peripheral blood	Confirmed genome-wide results of *NFκB2.* Methylation increased at probe 12, CpG sites 1, 5, and 6. Probe 5 (several important transcription factor binding sites) CpG sites 1, 2, and 5 increased in methylation
Zeng et al. (2012)	12 women (6 = exercise intervention, 6 = control), BC diagnosis	6 months of 150 minutes of moderate intensity treadmill exercise	HumanMethylation27 Beadchip of peripheral blood leukocytes/tumour samples	Methylation of 43 genes were altered, 6 were associated with overall survival (*IFT172*, *EPS15*, *GLUD1*, *PPP2R3A*, *MSX1*, *L3MBTL1*). Concordance between blood and tumour samples
Nitert et al. (2012)	15 men with a first-degree FH of T2DM, and 13 men without	6 months of 1 hour of spinning and 2x1 hour aerobic class per week	Genome wide analysis (MeDIP) of *vastus lateralis* muscle biopsy	Hypomethylation of 115 genes, and hypermethylation of 19 genes (independent of FH) - those with a FH, compared with no FH, had 38 differentially methylated genes (18↓, 20↑), in contrast to 65 before the

				intervention
Bryan et al. (2013)	64 sedentary men/women, mean age = 29 years	Psychologically tailored materials designed to increase PA participation over 12 months	HumanMethylation27 Beadchip of saliva samples	Post-intervention, self-reported PA score inversely correlated with methylation (after controlling for age, BMI and baseline $\dot{V}O_{2max}$)
Rönn et al. (2013)	15 men with a first-degree FH of T2DM, and 16 men without	6 months of 1 hour of spinning and 2x1 hour aerobic class per week	HumanMethylation450 Beadchip of subcutaneous adipose tissue of the right thigh	No difference in DNAme. at baseline between groups. Changes in methylation of 24 CpG sites in 18 candidate obesity genes, and 45 CpG sites in 21 candidate T2DM genes
Duggan et al. (2014)	Postmenopausal, healthy, overweight women aged 50-75 (70 = exercise intervention, 59 = control)	12 months of 3 supervised aerobic sessions (treadmill walking, cycling) per week, with encouragement to complete at least 2 more sessions at home	GM (LINE-1) of leukocytes	No sig. change in GM
Lindholm et al. (2014)	23 young participants (12 men, 11 women), not regularly performing intense exercise	3 months of one-legged knee extension exercise (4 sessions per week, 45 mins per session)	GM (LUMA) and HumanMethylation450 Beadchip of *vastus lateralis* skeletal muscle Validated using bisulfite pyrosequencing	LUMA revealed no global changes in GM. Training caused significant methylation changes at 4919 sites (839 changed by at least 5%) of 4076 differentially expressed genes
Rowlands et al. (2014)	18 obese, sedentary, middle-aged Polynesian men (5) and women (13) with T2DM	16 weeks of training, 3 days per week Endurance group: 40-60 minutes of cycle ergometer exercise (progressing from 65 to 85% of HR reserve during the 1st 2 weeks	DNAme:HumanMethylation450 Beadchip of *vastus lateralis* skeletal muscle miRNA: Affymetrix GeneChips 2.0 and TaqMan miRNA assay	Both training modalities caused global hypomethylation, with a number gene-specific DNAme. changes Endurance:

		Resistance group: 2-3 sets, 6-8 reps of 8 exercises using machine weights to fatigue (loads increased by 5% when participants could perform 10 reps)		↓ PM *NRF1* ↑ PM *FASN* ↓ TSS methylation *SLC27A4* ↓ GBM *PFKFB3*, *HDAC4*, *GSK3A* Resistance: ↓ upstream CpGi methylation *SLC2A4* ↓ genomic region methylation *ACSl1*, *LRP1*, *LRP10*, *SLC27A1*
Denham et al. (2015[b])	26 healthy young men not already engaging in intense training	4 weeks, 3x per week, 30sec sprints (athletics track) - sessions increased from 3/4/5 sprints in week 1 to 6/7/8 sprints in week 4	HumanMethylation450 Beadchip of whole blood leukocytes	Hypermethylation at 81.576 CpG sites Hypomethylation at 124,411 CpG sites. 19 sites exhibited a ≥20% change (8↑, 11↓) ↓ PM of *EGF* and ↓ mRNA ↓ PM of *UNG* and ↑ mRNA Methylation changes at various sites in *miR21* and *miR210* (the former did not remain sig. after FDR), with ↑ and ↓ mRNA, respectively
Denham et al. (2015a)	24 healthy young men not already engaging in intense training (13 cases, 11 control)	3 months (one human spermatogenesis cycle), 2x per week, 30sec sprints (athletics track) -	GM determined with ELISA, followed by HumanMethylation450 Beadchip	↓ GM (-6.63%)

		sessions increased from 3 sprints in week 1 to 6 sprints in week 12	of sperm DNA	7,509 CpG islands in 4,602 genes had sig. methylation changes 16 paternally imprinted genes were differentially methylated after training
Rodent Studies				
Gomez-Pinilla et al. (2011)	Adult male Sprague-Dawley rats (~3 months old)	Exercising rats given access to a running wheel for 7 days.	Bisulfite conversion and sequencing of hippocampal brain tissue Methylation assessed in 230bp region from -148 to +82 of *BDNF* exon IV promoter (14 CpG sites)	-148bp (associated with MeCP2 binding) was sig. ↓ methylated *BDNF* mRNA and protein ↑ by 41% and 30%, respectively
Abel & Rissman (2013)	10 exercising 46 day old C57BL/6J mice, 10 sedentary	10 mice were given access to a freely moving wheel for 7 days. 10 sedentary	Expression (PCR) of *DNMT1*, *DNMT3A* and *DNMT3B* in hippocampus and cerebellum	*DNMT1* showed a ↓ in expression of 19% and 14% in cerebellum and hippocampus *DNMT3A* and *DNMT3B* expression decreased by 17% and 19% in hippocampus
Elsner et al. (2013)	Male Wistar rats of different ages; 3 and 20 months old	Running on adapted motorised treadmill at 60% of $\dot{V}O_{2max}$ (acute bout: 20 mins; chronic: 2 weeks of 20 mins per day)	DNMT1 and DNMT3B ELISAs of hippocampal brain tissue	At baseline, 25% lower DNMT1 in aged vs young. No difference in DNMT3B Chronic exercise didn't alter either DNMT in either group

				Acutely, 30%↓ 1-h post in DNMT3B in young group compared with young sedentary control sedentary, returning to baseline by 18-h 45%↓ 1-h post in DNMT1 in young group compared with young sedentary control sedentary, returning to baseline by 18-h No acute changes in either DNMT in aged group
Carter et al. (2015) [abstract]	6 and 35-month old Fischer 34BN rats	Contractile activity (CA)	Tibialis anterior skeletal muscle. No details on methods for methylation analysis	↑ methylated DNA and 50% ↓ *PGC-1α* transcription in aged vs young muscle (at rest) 1.9x ↑ in DNMT3B protein in aged Following CA, *PGC-1α* transcription ↑ 2.5x in young muscle, but ↓ 7x in aged
Kanzleiter et al. (2015)	24 exercising 9 week old male C57BL/6J mice, 10 sedentary	Progressive motorised treadmill protocol from 30 to 50 mins per day, 5 days per week, 4 weeks	Reduced representation bisulfite sequencing (Illumina HiSeq2000) of genomic DNA isolated from quadriceps muscle (n=3/group) Transcriptional changes also quantified by Agilent 4x44k whole genome microarray and validated by PCR (n=6/group)	3692 differentially methylated CpGs (5% or > change), distributed among 2762 promoter regions 3020 differentially expressed genes, 361 (479 CpGs) associated with differential methylation. 66 hypomethylated genes = ↑ expression, 134 hypermethylated genes = ↓ expression

				MyoD and *myogenin* (plus 4 more) transcription factor binding sites were enriched
Lochmann et al. (2015)	6 exercising 3 month old female C57BL/KaLwRij mice, 6 sedentary	Rotarod training performed over 15 min, with 3.0cm rotarod increasing in speed from 15-35 RPM. After 1 days rest, an acute bout was performed at 35-45 RPM for 1 hour	Gene expression (PCR) of *PGC-1α* (*-a*, *-b*, *-c* transcripts, or total) in quadriceps muscle Tet-assisted bisulfite sequencing also performed	Total, '*-b*', and '*-c*' (promoter B), but not '*-a*' (promoter A) *PGC-1α* ↑ following acute bout DNA hydroxymethylation correlated to ↑ mRNA from promoter A, but DNAme. appeared to play no role in exercise-induced promoter B activation
Rodrigues et al. (2015)	4-6 male Wistar rats in each group: 1) stress group; 2) exercise and stress group; 3) control group	Exercise consisted of stimuli/task-free swimming. 60 mins per session, 5 days per week, 4 weeks	Imprint Methylated DNA quantification kit of genomic DNA isolated from brain (hypothalamus, frontal cortex region, PAG, hippocampus) Expression (PCR) of *DNMT1* and *BDNF*	No change in GM with exercise + stress in any tissue Stress alone caused a decrease in GM in hippocampus, cortex, and PAG compared with control ↓ *DNMT1* expression was observed in the cortex following exercise + stress
Kashimoto et al. (2016)	4 groups of 4-10 52 day old male Wistar rats (exercise, stress, exercise and stress, or control)	Exercise consisted of 60 mins swimming per day, 5 days per week (stimulus or task-free) from 53rd to 78th day of life	Imprint Methylated DNA quantification kit of genomic DNA isolated from brain (hypothalamus, frontal cortex region, hippocampus) Expression (PCR) of *DNMT1* gene	Exercise ↑ GM in hypothalamus. *DNMT1* expression was ↓ in hippocampus and hypothalamus in exercise and stress group, compared to just stress group

Appendix C – Informed Consent Form

INFORMED CONSENT FORM

Title:

Principal Investigator: Steven Horsburgh

I have carefully read and understood the Participant Information Sheet.	☐
I have had an opportunity to ask questions and discuss this study and I have received satisfactory answers.	☐
I understand I am free to withdraw from the study at any time, without having to give a reason for withdrawing, and without prejudice.	☐
I agree to take part in this study.	☐
I would like to receive feedback on the overall results of the study at the email address given below. Email address…………………………………………………………………	☐

Signature of participant... Date......................... (NAME IN BLOCK LETTERS)...
Signature of Parent / Guardian in the case of a minor ....
Signature of researcher... Date......................... (NAME IN BLOCK LETTERS)...

FOR USE WHEN TISSUE IS BEING REMOVED AND STORED

Title:

Principle Investigator: Steven Horsburgh

Participant ID:

I agree that the following tissue or other bodily material may be taken and used for the study:

Tissue/Bodily material	Purpose	Removal Method
Venous Blood	DNA methyltransferase DNA methylation	Venepuncture

I understand that if the material is required for use in any other way than that explained to me, then my consent to this will be specifically sought. I understand that I will not receive specific feedback from any assessment conducted on my samples, but should any kind of abnormality be discovered then the investigator will contact me.

I understand that the University may store this tissue in a Licensed Tissue Bank only for the duration of the study, it will then be destroyed.

Method of disposal:

Clinical Waste ☐

Other ☐

Signature of participant... Date........................

(NAME IN BLOCK LETTERS)..

Signature of Parent / Guardian in the case of a minor

....

Signature of researcher... Date........................

(NAME IN BLOCK LETTERS)..

If other please specify...

I consent to the University distributing this tissue to partners in this research study, outside of the University, for further testing (please tick the box if you agree). ☐

Appendix D – Methyl-donor Food Frequency Questionnaire

Supplementary material 1 Food-Frequency Questionnaire

Name:.................................

Date: / /

In this food-frequency questionnaire we inquire about your food habits of the **past 3 months**. In the following table a variety of food products (food groups) is listed. Please describe (as exact as possible) how often you eat or drink the listed products and indicate the average daily portion. Consider also the meals taken away from home.

How often (frequency)?

In the column with the heading 'How often do you consume the following product?' there are 6 possible answers.

- Never or less than once per month
- 1-3 days per month
- 1 day per week
- 2-4 days per week
- 5-6 days per week
- Every day

Indicate your choice by filling in the circle near the answer that is most suitable for you.

How much?

In the column with the heading 'What is the average portion per day?' 3 to 5 portion size options are given.

In the column with the heading 'Example portion sizes', a number of directive weights and measures are given. These can help you to quantify the average portion sizes. Indicate your choice by filling in the circle near the answer that is most suitable for you.

Which type do you usually use?

In the last column you should indicate for some food products (food groups) the type you usually use. Please choose only one answer, unless options are equally frequent. In the latter case you may indicate more options.

In case you would fill in the wrong option, you may cross it out and color another option. Please indicate in such case the right answer with an arrow. Make sure you always fill in something, even when you consume a product rarely or never. In such case, choose the option 'Never or less than once per month' without indicating a portion size or type.

Food groups	How often do you consume the following product?	What is the average portion per day?	Example portion sizes	Which type do you usually use?
Coffee/tea	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 125 mL or less ○ 125 - 250 mL ○ 250 - 375 mL ○ 375 - 500 mL ○ 500 mL or more	1 cup = 125 mL 1 beaker = 225 mL	○ Coffee ○ Tea
Beer/wine	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 200 mL or less ○ 200 - 400 mL ○ 400 - 600 mL ○ 600 - 800 mL ○ 800 mL or more	1 bottle/glass beer = 250 or 330 mL 1 can = 330 or 500 mL 1 glass of wine = 125 mL	○ Alcohol free beer ○ Regular beer (*pils, Palm,...)* or strong beer *(Duvel, trapist,...)* ○ Wine
Soup	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 200 mL or less ○ 200 - 400 mL ○ 400 - 600 mL ○ 600 mL or more	1 bowl = 250 mL 1 beaker = 225 mL	
Fruit juice	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 150 mL or less ○ 150 - 300 mL ○ 300 - 450 mL ○ 450 mL or more	1 glass = 150 mL 1 carton = 200 mL 1 bottle *(Looza)* = 200 mL	○ Orange juice ○ Grapefruit juice ○ Other
Vegetable juice	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 150 mL or less ○ 150 - 300 mL ○ 300 - 450 mL ○ 450 mL or more	1 glass = 150 mL 1 carton *(V8)* = 330 mL 1 bottle *(Looza)* = 200 mL	
Softdrinks *(cola, lemonade,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 150 mL or less ○ 150 - 300 mL ○ 300 - 450 mL ○ 450 mL or more	1 glass = 150 mL 1 can = 330 mL 1 bottle = 200 mL	○ Cola ○ Other

Soy milk/ drinks and yoghurt drinks *(Dan 'Up, Fristi)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 125 mL or less ○ 125 - 250 mL ○ 250 - 375 mL ○ 375 mL or more	1 glass = 150 mL 1 beaker = 225 mL 1 soy drink = 250 mL 1 bottle *(Dan'Up)* = 600 mL	○ Soy milk/ soy drinks ○ Yoghurt drinks
Milk/ chocolate milk	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 125 mL or less ○ 125 - 250 mL ○ 250 - 375 mL ○ 375 mL or more	1 cup = 125 mL 1 beaker = 225 mL 1 bowl = 250 mL 1 glass = 150 mL 1 carton = 200 mL	
Yogurt, cottage cheese, white cheese, and curd	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 125 g or less ○ 125 - 250 g ○ 250 - 375 g ○ 375 g or more	1 pot yoghurt = 125 g 1 pot cottage cheese = 200 g 1 cup = 125 g 1 dish = 150 g 1 bowl = 250 g	○ Yogurt ○ Yogurt with fruit ○ Cottage cheese/ white cheese/curd
Eggs	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 1 piece or less ○ 2 pieces ○ 3 pieces or more		
Cheese spread *(type Philadelphia)* and melted cheese/cheese spread *(Kiri, Ziz,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 15 g or less ○ 15 - 30 g ○ 30 - 45 g ○ 45 g or more	15 g per slice of a big bread 10 g per slice of a small bread	○ Cheese spread type Philadelphia ○ Melted cheese/cheese spread
Feta, goat cheese, mozzarella, and Parmesan cheese	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 15 g or less ○ 15 - 30 g ○ 30 - 45 g ○ 45 g or more	15 g goat cheese per slice of a big bread 1 small block of feta = 5 g 1 Mozzarella ball = 125 g 1 tablespoon of grated Parmesan = 10 g	○ Feta/goat cheese ○ Mozzarella/ Parmesan
Other cheese *(Brie, Camembert, Roquefort)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week	○ 15 g or less ○ 15 - 30 g ○ 30 - 45 g ○ 45 g or more	15 g per slice of a big bread 10 g per slice of a small bread	

	○ 5-6 days per week ○ Every day			
Hard cheese/ semi-hard cheese *(Gouda, Emmentaler, Cheddar, Gruyère,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 20 g or less ○ 20 - 40 g ○ 40 - 60 g ○ 60 g or more	1 slice of cheese (10x10cm) = 25 g	
Pâté/liver sausage	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 20 g or less ○ 20 - 40 g ○ 40 - 60 g ○ 60 g or more	20 g pâté per slice of bread	
Other meat products *(ham, salami, chicken ham,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 20 g or less ○ 20 - 40 g ○ 40 - 60 g ○ 60 g or more	15 g per slice of bread	
Canned fish, smoked fish, and salted fish	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 50 g or less ○ 50 - 100 g ○ 100 - 150 g ○ 150 g or more	1 spoon of canned tuna/salmon = 25 g 1 slice of smoked salmon = 30 g 1 young herring = 50g 1 drained can of mackerel/ sardines = 120 g	
Breakfast cereals: muesli *(normal and crispy)* and oatmeal	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 30 g or less ○ 30 - 60 g ○ 60 - 90 g ○ 90 g or more	1 bowl = 40 g	
Breakfast cereals: All Bran *(Kellogs)* and wheat bran	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 30 g or less ○ 30 - 60 g ○ 60 - 90 g ○ 90 g or more	1 bowl = 40 g	○ All Bran ○ Wheat Bran
Breakfast cereals: Special K and others *(Type Smaks, Honey Pops, Frosties)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per	○ 30 g or less ○ 30 - 60 g ○ 60 - 90 g ○ 90 g or more	1 bowl = 40 g	○ Special K ○ Others

	week ○ 5-6 days per week ○ Every day			
Rusk, crisp bread, and rice wafer	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 10 g or less ○ 10 - 20 g ○ 20 - 30 g ○ 30 g or more	1 piece = 8 g	
Fantasy bread *(sugar bread, raisin bread, brioche)* and Danish pastry	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 50 g or less ○ 50 - 100 g ○ 100 - 150 g ○ 150 g or more	Croissant = 50 g 1 Danish pastry = 100 g 1 slice of bread = 25 g	○ Fantasy bread ○ Danish pastry
Types of white bread (*bread roll, baguette*) and yeast bread	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 210 g ○ 210 g or more	½ baguette = 120 g 1 slice of a big bread = 30 g 1 slice of a small bread = 20 g 1 slice of yeast bread = 45 g	
Types of brown bread *(whole grain, multigrain, rye, brown baguette)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 210 g ○ 210 g or more	½ baguette = 120 g 1 slice of a big bread = 30 g 1 slice of a small bread = 20 g	
Dried fruit	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 15 g or less ○ 15 - 30 g ○ 30 - 45 g ○ 45 g or more	1 prune, apricot, date = 8 g 1 tablespoon raisins = 12 g	
Orange, mandarin, grapefruit, kiwi, strawberries, and banana	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 100 g or less ○ 100 - 200 g ○ 200 - 300 g ○ 300 g or more	1 mandarin = 60 g 1 kiwi = 75 g 1 banana, orange = 130 g 1 grapefruit = 210 g 1 strawberry = 15g	○ Orange, mandarin, and grapefruit ○ Kiwi ○ Banana ○ Strawberry

Chocolate	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 25 g or less ○ 25 - 50 g ○ 50 - 75 g ○ 75 g or more	1 Mignonette = 10 g 1 individual bar of chocolate = 50g 1 bar of a big package = 25g Easter egg or figure in chocolate = 50 g	
Salty snacks *(crisps, tortillas, salted biscuits)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 20 g or less ○ 20 - 40 g ○ 40 - 60 g ○ 60 g or more	1 small packet of crisps = 30 g 1 Tuc biscuit = 3 g	○ Crisps/tortilla ○ Salted biscuits
Nuts/seeds en nut-seed-paste *(peanut butter and sesame paste)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 15 g or less ○ 15 - 30 g ○ 30 - 45 g ○ 45 g or more	10 cashews = 20 g 1 tablespoon nuts = 25g 1 tablespoon peanut butter = 15 g	
Olives en sundried tomatoes	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 15 g or less ○ 15 - 30 g ○ 30 -45 g ○ 45 g or more	5 olives = 20 g 1 sundried tomato = 12 g	○ Olives ○ Sundried tomatoes
Cereal bar, granola bar, dry biscuits and biscuits with chocolate	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 20 g or less ○ 20 - 40 g ○ 40 - 60 g ○ 60 g or more	1 Special K bar = 20 g 1 petit beurre biscuit = 13 g 1 Evergreen cookie = 40 g 1 chocolate chip cookie = 20 g	○ Cereal bar, granola bar ○ Biscuits without chocolate ○ Biscuits with chocolate
Cake/muffin	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 20 g or less ○ 20 - 40 g ○ 40 - 60 g ○ 60 g or more	1 slice of cake = 30 g 1 cupcake = 30 g 1 muffin = 75 g	○ Cake ○ Muffin
Pancakes	○ Never or less than once per month ○ 1-3 time per	○ 60 g or less ○ 60 - 120 g ○ 120 - 240 g ○ 240 g or more	1 pancake = 60 g	

	month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day			
Potatoes *(cooked, steamed, baked, mashed...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 75 g or less ○ 75 - 150 g ○ 150 - 225 g ○ 225 - 300 g ○ 300 g or more	1 cooked potato = 50 g 1 tablespoon of mashed potatoes = 50 g 20 French fries or 3-4 croquettes = 100 g	
Rice and other grains *(bulgur, quinoa, couscous)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 25 g uncooked or less = 62 g cooked or less ○ 25-50 g uncooked = 62 -125 g cooked ○ 50 -75 g uncooked = 125 - 187 g cooked ○ 75 - 100 g uncooked = 187-250 g cooked ○ 100 g uncooked or more = 250 g cooked or more	60 g uncooked rice = 150 g cooked rice 1 tablespoon cooked rice = 25 g 1 bag of rice for 2 persons = 125 g uncooked rice	○ White rice ○ Brown rice ○ Other grains
Pasta *(penne, spaghetti ,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 40 g uncooked or less = 100 g cooked or less ○ 40 - 80 g uncooked = 100 - 200 g cooked ○ 80 - 120 g uncooked = 200 - 300 g cooked ○ 120 - 160 g uncooked = 300 - 400 g cooked ○ 160 g uncooked or more = 400 g cooked or more	50 g uncooked pasta = 125 g cooked pasta 1 tablespoon cooked pasta = 25 g	○ White pasta ○ Wholemeal pasta
Crustaceans, shellfish *(scampi, crab, shrimps, lobster, mussels, ...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 50 g or less ○ 50 - 100 g ○ 100 - 150 g ○ 150 g or more	1 scampi = 15 g 1 portion mussels with shells = 1200g	
Fish/fish sticks	○ Never or less than once per month ○ 1-3 time per	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g	1 fish stick = 30 g 1 serving of fish = 175 g	○ Fish ○ Fish sticks

	month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 240 g or more		
Beef *(steak, roast beef,…)* and veal *(leg, steak,…)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 steak = 175 g 1 slice of roast beef = 40 g 1 vealsteak/leg = 150 g	
Pork *(bacon, pork chop,...)* and lamb *(leg, chop,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 portion of pork belly = 75 g 1 hamburger = 110 g 2 lamb chops (boneless) = 150 g	○ Pork ○ Lamb
Other meat *(horse, rabbit, wild,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 rabbit leg (boneless) = 160 g Dear steak = 100 g	○ Horse ○ Other
Poultry *(chicken and turkey)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 fillet = 160 g 1 chicken leg = 160 g 1 chipolata = 70 g	
Organ meats *(liver, kidney)* and tongue	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 slice of foie gras = 40 g 1 porcine kidney = 140 g	○ Liver ○ Kidney ○ Tongue
Vegetarian products *(tofu, quorn, burgers)* NO vegetable burgers,...	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 50 g or less ○ 50 - 100 g ○ 100 - 150 g ○ 150 g or more	1 large burger = 95 g 1 small burger = 55 g 1 box of quorn minced meat = 175 g	
Raw vegetables	○ Never or less than once per month	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g	1 serving of leafy vegetables = 50 g 1 tablespoon shredded carrots	

	○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 180 - 240 g ○ 240 g or more	= 20 g 1 tomato = 150 g	
Cabbages *(Brussels sprouts, cauliflower, red cabbage, savoy)* and spinach	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 tablespoon Brussels sprouts = 40 g 1 tablespoon red cabbage = 25 g 1 tablespoon cauliflower = 30 g 1 tablespoon spinach = 45 g	○ Cabbages ○ Spinach
Other winter vegetables *(Belgian endive, broccoli, beet, leek, carrot, turnip,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 chicory = 85 g 1 tablespoon cooked vegetables = 30 g	
Summer/ Spring vegetables (*corn, pepper, soy beans, asparagus, mushroom,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 asparagus = 50 g 1 tablespoon of cooked vegetables = 30 g	
Legumes *(chickpeas, beans, green beans, lentils, peas,...)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 60 g or less ○ 60 - 120 g ○ 120 - 180 g ○ 180 - 240 g ○ 240 g or more	1 tablespoon peas/ green beans = 25 g 1 tablespoon chickpeas/lentils/ beans = 35 g	
Sauces *(warm and cold)*	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 12 g or less ○ 12 - 25 g ○ 25 - 50 g ○ 50 g or more	1 tablespoon mayonnaise = 25 g 1 tablespoon gravy = 12 g 1 tablespoon white sauce = 20 g	

Lasagna, spaghetti, and pizza	○ Never or less than once per month ○ 1-3 time per month ○ 1 time per week ○ 2-4 days per week ○ 5-6 days per week ○ Every day	○ 200 g of less ○ 200 - 400 g ○ 400 - 600 g ○ 600 g or more	1 frozen pizza = 400 g 1 pre-packed lasagna = 400 g	

Do you occasionally use haddock liver, caviar or seaweed? ○ Yes ○ No

In case your answer is yes, please clarify how much you use of these products.

- Haddock liver:..
- Caviar:...
- Seeweed:..

Do you use **enriched products** like breakfast cereals enriched with folic acid (see the package for information).

○ Yes ○ No

In case your answer is yes, please specify the product: ....

Do you use **food supplements (vitamins and/or minerals)**? Fe. Supradyn, 1 tablet/day

Fe. Omnibionta, 1 tablet/day

○ Yes ○ No

In case your answer is yes, leave the product name and your daily use: ………………………………………………

Appendix E - Sample Size Estimation

Sample size was calculated using a spreadsheet accessed from the Boston University and Boston Medical Centre Research Compliance website ("Sample-Size Calculations", 2014).

The sample size calculation for the studies presented in chapters 4 and 6 was based on pilot data (see chapter 3), whereby rIL-6 caused a significant increase in the nuclear concentrations of DNMT3B. 10 participants were recruited based on this, to account for drop out.

	A	B	C	D	E	F	G	H	I
1	**I - Sample Size Calculations for Means**								
2		**Anticipated Values**							
3		**Mean**	**Stan. Dev**						
4	***Group 1***	365	11		Difference in means		34.24658	%	
5	***Group 2***	490	90						
6									
7	The cells in the table below show the estimated number of subjects needed in each								
8	group in order to demonstrate a statistically significant difference at "p" values								
9	ranging from 0.10 - 0.01 and at varying levels of "power".								
10		Power is the probability of finding a statistically significant difference at							
11	a given "P" value with the specified number of subjects in each group.								
12									
13			**Sample Size Needed in Each Group**						
14	**alpha level**			**Power**					
15	**("p" value)**		**95%**	**90%**	**80%**	**50%**			
16	**0.10**		6	5	3	1			
17	**0.05**		7	6	4	2			
18	**0.02**		8	7	5	3			
19	**0.01**		**9**	8	6	3			

For the final study presented in chapter 7, sample size was estimated based on previous studies that had investigated $\dot{V}O_{2max}$ changes following a training period. Zoladz, Pilc, Majerczak, Grandys, Zapart-Bukowska, & Duda (2008) reported that $\dot{V}O_{2max}$ increased from 34.7 (± 0.9) to 35.9 (± 1) $ml \cdot kg^{-1} \cdot min^{-1}$ following 5 weeks of aerobic training on a cycle ergometer in recreationally active young men.

	A	B	C	D	E	F	G	H	I
1	**I - Sample Size Calculations for Means**								
2		**Anticipated Values**							
3		**Mean**	**Stan. Dev**						
4	***Group 1***	34.7	0.9		Difference in means		3.458213	%	
5	***Group 2***	35.9	1						
6									
7	The cells in the table below show the estimated number of subjects needed in each								
8	group in order to demonstrate a statistically significant difference at "p" values								
9	ranging from 0.10 - 0.01 and at varying levels of "power".								
10		Power is the probability of finding a statistically significant difference at							
11	a given "P" value with the specified number of subjects in each group.								
12									
13			**Sample Size Needed in Each Group**						
14	**alpha level**			**Power**					
15	**("p" value)**		**95%**	**90%**	**80%**	**50%**			
16	**0.10**		14	11	8	3			
17	**0.05**		16	13	**10**	5			
18	**0.02**		20	16	13	7			
19	**0.01**		22	19	15	8			

Dengel, Pratley, Hagberg, Rogus, & Goldberg (1996) demonstrated that 10 months of aerobic treadmill training increased $\dot{V}O_{2max}$ from 2.58 (± 0.19) to 2.91 (± 0.18) l·min^{-1}.

	A	B	C	D	E	F	G	H	I
1	**I - Sample Size Calculations for Means**								
2		**Anticipated Values**							
3		**Mean**	**Stan. Dev**						
4	***Group 1***	2.58	0.19		Difference in means		12.7907	%	
5	***Group 2***	2.91	0.18						
6									
7	The cells in the table below show the estimated number of subjects needed in each								
8	group in order to demonstrate a statistically significant difference at "p" values								
9	ranging from 0.10 - 0.01and at varying levels of "power".								
10		Power is the probability of finding a statistically significant difference at							
11	a given "P" value with the specified number of subjects in each group.								
12									
13			**Sample Size Needed in Each Group**						
14	**alpha level**			**Power**					
15	**("p" value)**		**95%**	**90%**	**80%**	**50%**			
16	**0.10**		7	5	4	2			
17	**0.05**		**8**	7	5	2			
18	**0.02**		10	8	6	3			
19	**0.01**		11	9	7	4			

Finally, 12 months of moderate intensity aerobic training in a sample of overweight men elicited a significant elevation in $\dot{V}O_{2max}$ from 33.4 (± 2.9) to 42.5 (± 5.1) ml·kg^{-1}·min^{-1} (Thompson et al., 1997). 10 men were recruited for each group (exercise and control) to allow for potential drop outs.

	A	B	C	D	E	F	G	H	I
1	**I - Sample Size Calculations for Means**								
2		**Anticipated Values**							
3		**Mean**	**Stan. Dev**						
4	***Group 1***	33.4	2.9		Difference in means		27.24551	%	
5	***Group 2***	42.5	5.1						
6									
7	The cells in the table below show the estimated number of subjects needed in each								
8	group in order to demonstrate a statistically significant difference at "p" values								
9	ranging from 0.10 - 0.01and at varying levels of "power".								
10		Power is the probability of finding a statistically significant difference at							
11	a given "P" value with the specified number of subjects in each group.								
12									
13			**Sample Size Needed in Each Group**						
14	**alpha level**			**Power**					
15	**("p" value)**		**95%**	**90%**	**80%**	**50%**			
16	**0.10**		4	4	3	1			
17	**0.05**		5	4	3	2			
18	**0.02**		7	5	4	2			
19	**0.01**		**7**	6	5	3			

Appendix F – Physical Activity Readiness Questionnaire (PAR-Q)

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	**Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	2.	**Do you feel pain in your chest when you do physical activity?**
☐	☐	3.	**In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4.	**Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5.	**Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6.	**Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7.	**Do you know of any other reason why you should not do physical activity?**

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME ____________________

SIGNATURE ____________________ DATE ____________________

SIGNATURE OF PARENT or GUARDIAN (for participants under the age of majority) ____________________ WITNESS ____________________

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

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INSTRUCTIONS FOR USING THE FOOD DIARY

- Everything that you eat and drink over the course of the day should be recorded.

- Do not forget to record second helpings and snacks between meals.

- Eating Out – most people eat foods away from home each day - please do not forget to record these. Take your diary with you wherever possible.

- Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

e.g. Cheese

Cheese, cheddar (Pilgrim's Choice) - 30g

- Start a new page in your diary for each day, and record each item on a separate line. Record the time of day in the first column of each line.

e.g. 10:30 am - McVities Digestive Biscuits (x2) - 50g

- The space provided at the foot of each page for general comments is for you to give any further information about your diet

DAY 1
Date: /   /

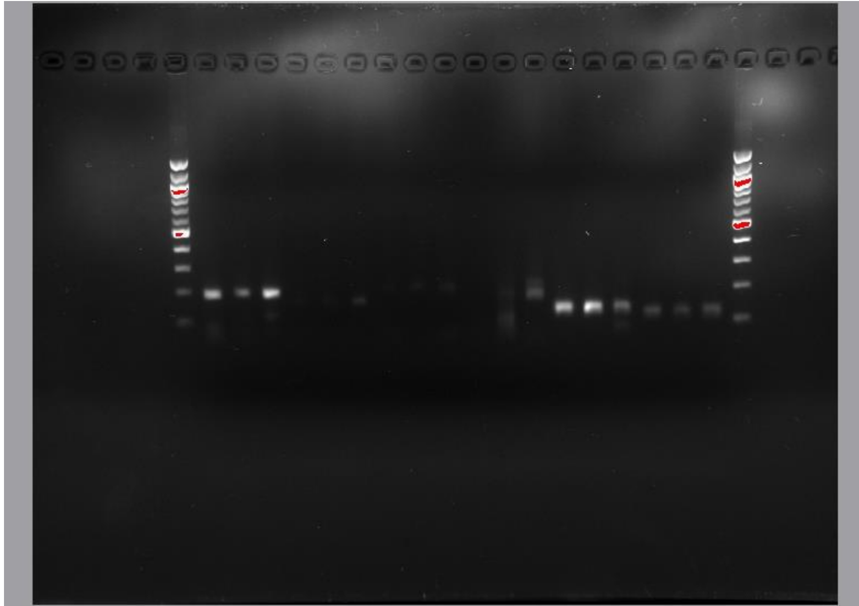
Please use a separate line for each item eaten; write in weight of plate; leave a line between different ‘plate’ entries.

A	B		C	D	E	F	10 **Office Use**
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, dried, canned -cooked: boiled, grilled, fried (incl. type of fat), roasted etc.	Weight Served	Weight of Leftovers	Actual Weight
am/pm	home	away			(gms)	(gms)	(gms)

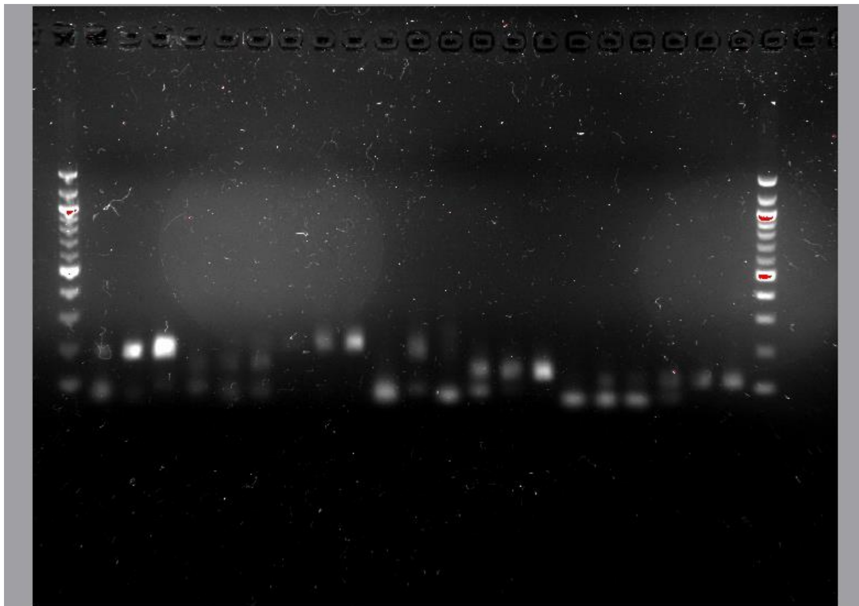
GENERAL COMMENTS

Appendix H – Gel Electrophoresis Images

The image below shows all potential primer pairs performed in triplicate. Gel electrophoresis was used to check for product specificity and dimerization.



Optimal annealing temperatures were then tested (from 55 ºC to 59 ºC).



Appendix I – High Resolution Melting Primer Design

IL6

Primer No.	Forward Sequence	Reverse Sequence	Position
1	GGAGAGTATTAAAAATGAAATTATT TAGTT	ATAACCTCTATTAAACATTTACTCAA ATTT	Promoter
2	GGAAAGAGAATTTGGTTTAGGAGT	AAAATAAAATCATCCATTCTTCACC	1st exon
3	GTTGGGGAAAGTGAGGTTATTTATT	ACACTAAAAATAAAACCATCCAACC	Promoter

Selected assay mapped onto mock bisulfite converted gene sequence

```
TTATAATTGGTTTTTTTGTTTTGTTAAGAAGAGATTTTTTTAAGATCGATAAAATAGTGATTTTTGTTGGGTA
TTTATTTAAGTTTTGTTTCGTGTATAATATTTTAGGATTCGTT (TGTTGGGGAAAGTGAGGTTATTTATTTTT
TATCGATTGTTTAAATAGAGACGGTGGTTTTTTGTCGCGGTGGGATTTTTTGGTGTGAAAATGAAAATGGGGG
AGGA) TGGTTGGATGGTTTTATTTTTAGTGTTTTTTTGTTTTAAGTAACGTAGATATTTTTGAATTAAGTTTT
TTTTTTTGTATGAAACGAAGTTATTGTTTTTAGTTTCGTAGTCGTGTATTGTGATTCGTTTATGTAATGTGAG
TTTGTTTTTATTTAATTATTTATTTTTTAGAAAATATAATTTAATAAATTTTAAGTATTTGAATTGAGTGATA
TAATATAATATATAGATTTATTTTTTTAATAAAAAGTGTTTTTTAAATAGGAAGTTCGTGTTTATGATAAAAT
GTTTAAATAGGATATTAGGGTATGTAGGGAAAAGTGTTTTTTTTTTTTTTTGGTTTTTTTTTTTAGAGGTAGT
ATTTAATTGTTTGTATTTTTAATTAAATTTTTTGTTATTTTAGTTTGGGAAAGGTTTTTTCGTTTTTTTTAGTT
GTTTGATTTTGGTTGTTATATTAAGGATTTGTGTTTTTGTTTATTTATTTATAAAACGGGGGTAATAATAGTT
TTTTTTTTTTAGGGTTGTTGTGAAGATGGAACGTGATGTAGTGTAAAAAATTTTTGGCGTATAGTAATTTTAT
AATATATGTTAGTTTTTTGAATATTTTTTGGTTTAGAGTTTTTGTTTTGGAGAGTTTTTTTAGTTCGGTTATA
TCGGTTTATTTGGTGTTTTGTTTATGAAGGAATTATGAATGAATTAGGTTTTTGTGTTAGTAATGAGTTGGTT
TTTTAATTATTTTTTTTTAAAAGAAATGAGATTTTATGTTTTGTTGAAAATGTTTTTTTGTTGGGGATGTTAA
AGGAGGATTTTGTGGTATTTTGTAGAGTTTGTGTTTATCGTTTTTTTTTTTTGTAAGTTTTGATTTGAAATTA
AAAGAAGTTAGAAGGAAATTACGGACGTAGGTACGGTTTTAGGTTTTGAATTTGTTTTCGCGTCGGTATTTAA
GAATTTTTTATTGGGTTAAGTTGGTTTAGAAGTTTTTTTTTTTTAGGATGGTGTTTTTTGTAGGAGGATTTTT
GGTTTTATGTAAATGGGTTTTAGATTAGGTTGTTTTTTTAGTTTTTAGAGATTTAGATAGTTTTGAGATGGTT
TTAGGTTGAAATTAGATTTTTGTATAATATTAAAATATTTATTTATTAAATATGGTGTTATTT
```

1st Exon

(CpGi)

Primers

Product

CG

NFκB2

Primer No.	Forward Sequence	Reverse Sequence	Position
1	GGTTTTGAGTTTTGTTGGTAGG	AAATCTAAATCCAACCACTCACC	1st exon
2	TTAGGGGTTTGAATTTAGTTATTTAT	ACCCTAAACCCTACTAACAAAC	1st exon

Selected assay mapped onto mock bisulfite converted gene sequence

GAAGTTATTTTTGCGGTCGGTTTCGGGCGTTTTTTGGCGGTTTTTTTGGGTTCGCGAGTAGATTTTATTCGGT
TTCGGTTTTTTTTTTCGTTTCGTTTTATTAGTTTTAAGGTTTCGGGAGAGATAGTTCGGATTTCGAATTTTTTT
AGAAGTGATAGGTTTTTAGCGGGTTAGGGTTTAGGAGTTTTGTTTAGGAGAAGGGAAATGAAGTGTTTATTTA
TTAAGGAGATTTGGAATATTAGAATTGAAAAAGGTATTTAGAGTTTAAATAGTTTAAATTTTTATCGATGTGG
GAAATCGTTTTTTTTTTTTTAGTTTTAGGTTGGAATGTAGTGAGGGGATTTTGGTTTATTGTAATTTTTGTTTT
TCGGGTTTAAATTATTTTTTTGTTGTAGTTTATCGAGTAGTTGGGATTATAGGCGGTTATTATTACGTTCGTT
TAATTTATGTATTTTTAGTAGAGTTGGGGGTTTTATTACGTTGGTTAGGTTGGTTTCGAATTTTTGATTTTAG
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ATGGTTTATTGTAATTTCGAATTTCGGGGTGCGTCGATTTTTTGATTTTTTTGAGTAGTTGGGGTTATAGGTG
TATATTATTATATTTGGTAAATTTTAAAATTTTTAGTGGAGATGGATTTTATTGCGTTGTTTAGGTTGGTTTT
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TATTGTATGTTGTCGAATTGTTTTTTATAGATTTTTTAATTTTTATTTGAATATTTTTAGAGGTAGCGAGGTT
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TTTTCGGTTGTGTGTAGTTGTTGTTTTTTAGTTTTAGGCGTTGTTCGTTAAAGGTTTTTTTTTTTTTT (TTTTT
TTTGTTTCGGTTATTTAGGGGTTTGAATTTAGTTATTTATCGTGGCGGCGGTTTTGGTTGCGGTTTTTTTTCG
CGTTCGATTTATTTGGGATCGGTCGCGATATTTCGTTTGTTAGTAGGGTTTAGGGTCGGGAAAGTTTTTTTTC
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TTTAATTTTGGATTGGCGTAGTTAATTCGGAGCGATTCGGGCGCGTTTTTGAGAGGGGATAGGTTTATTTAGG
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TTTAGCGTTATTTCGGGTCGGGAGTTTTCGTTGTTTTTCGGTTTTGGTTGCGTTTAACGTTTGGTTTTTTTTG
AAGCGGGGGATTTTTAGGGGGAAGTCGACGGTTTTAGTTTTCGGGGTTTTTATTTTGAGCGGCGCGTTGTCGG
GAA) GTTAGCGGAAGAGGAAGGAAGTTATTTTTGCGGTCGGTTTCGGGCGTTTTTTGGCGG

1st Exon

(CpGi)

Primers

Product

CG

ASC

Primer No.	Forward Sequence	Reverse Sequence	Position
1	TAAGAAGTTTAAGTTGAAGTTGTTGT	ACCATCTCCTACAAACCCATATC	1st exon
2	TTTTAGTATGTGGAATTGAGGGAGT	ATTAAACACCTAAACTTAAAACCTC	1st exon

Selected assay mapped onto mock bisulfite converted sequence

```
AGAGTATTGTTTTTTTATCGTGGTTTTTGTTTGTTTGTTTTTGTTTTTGTTTTGAGATGGAGTTTCGTTTTTGT
TGTTTAGGTTGAAGTGTAATGGTGTGATTTTAGTTTATTGTAATTTTTATTTTACGGGTTTAGGCGATTTTTT
TGTTTTAGTTTTTTTAGTAGTTGGGATTATAGGTATTTGTTATTACGTTCGGTTAATTTTTGTATTTTTAGTA
GAGATGGGGTTTTATTTTGTTGGTTAGGTTGGTTTTAAATTTTTGATTATTTATTTCGTTTTTTTAAAATGTT
GGGATTATAGGTATGAGTTATCGTGTTCGGTTTATTTTGGTTGTTTTGTTTGTTTTTGAGATAAGGTGTTTGT
TGTCGAGTGGTATAGAGTGTAGTGGTATAATTATAGTTTATTGTATTTTTAATTTTTTGGGTTTAAGTGATTT
TTCGGTTTTAGTTTTTTAAAGTATTGAGATGATATGCGTGATGAGTTATTGTATTTAGTTATGTTGGAAGTTT
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TAGTTAAGGTTTTCGATGTTTATAGGTTTTATATTTTAGAGAGGGATGAATGTTTAATAAGTATATAAACGTA
TAATATGTTAGGGTCGTATGATTATAAGGAATAGTGATTGTTATAATTTAGATGAGAGGGAAAAATAAAGGAT
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AGGAAGTATTTGGTAAGGTTTCGGGAGTTATCGGGTTGGTTATGTTATGTTGGAATATTTATAAGTATCGGAG
GGTTATTTTTATGTCGTAGAAAATGAAATTGAAGTTTAGAGAGATTTGTATTTTTTGTTTTTTTGTATAATTT
ATTTTTTTTTTAGTATGTGGAATTGAGGGAGTTTTACGTTTTTAG (TTGTTATGATTTTAAGATTTTACGATAT
GTGGGAGAGGATTTTAAGGTTCGGGGAATCGCGGAGGTTTCGGGGTTTTAGAAATTCGAGGTTTTAAGTTTAG
GTGTTT) TAATAAATTTAGTGAGAGTTAGTTTAGGTTTTCGGTTTGTATTCGTTGGTGTAAGTTTAGAGATAA
GTAGGTGTTATTTATGAGTTTTTTTGCGGTTTTTTTTCGGGTTTTATTTCGTAGGTTAGTTGGAGGGCGCGAT
TTTGGCGTTTTTCGACGGTTTGGGGTTTTAATTTAGAGGTTTGGGTGGAGGGGATTAAGGGTGTAGTAAGGA
A (GTGTTTTTTGTTGGAGGGTAACGGATCGGGGCGGGGAGTCGGGAGATTAGAGTGGGAGGAAGGCGGGGAGT
TTAGGTTTCGTTTCGGAGTCGATTTTTTTTTGGTCGGTGGTTGTAGCGGGGTGAGCGGCGGTAGCGGTCGGGG
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GAAGTTTAAGTTGAAGTTGTTGTCGGTGTCGTTGCGCGAGGGTTACGGGCGTATTTCGCGGGGCGCGTTGTTG
TTTATGGACGTTTTGGATTTTATCGATAAGTTGGTTAGTTTTTATTTGGAGATTTACGGCGTCGAGTTTATCG
TTAACGTGTTGCGCGATATGGGTTTGTAGGAGATGGTCGGGTAGTTGTAGGCGGTT) ACGTATTAGGGTGAGT
CGTTTTCGTTTTTTTTTTATTTCGTTTTTTTTTTTTTATTTATATTAGCGTTTATTTCGCGGGTTTTTTCGTTTT
TTGTTTTTTTTTATTTTTAAATAAAGTTGTTTTATCGGAAAGGAGGTTTTTTACGTTTGGTTTATCGATTAACG
GGATTTCGGTTTTACGGCGGGAAGGGAAGGGAAGGGGATTATTTGGTTTATATTTTTTTAGGTTTTGGAGTCG
CGCTAGTTGGGATTTAGGTTTTTTTTTTAGTCGGTAGTTAAGTTAG
```

1st Exon

(CpGi)

Primers

Product

CG

Appendix J – Gene Expression Primer Design

DNMT3A

Transcript	Forward Sequence	Reverse Sequence	Probe #
All (common assay)	TTGATGAGCGCACAAGAGAG	CCACAGGAGATGCAGATGTC	29
001	GAAGCCTCAAGAGCAGTGGA	TGTTCTTTGCCCGCTTCT	46
002	GCTGCACCTGGCCTTATG	CGTCTTTCAGGCTACGATCC	1
011	GAAGCCTCAAGAGCAGTGGA	TGTTCTTTGCCCGCTTCT	46
012	GCGCTTTCTCATACATTATCACAT	GCACATATGCAAAACAACCTG	81
013*			

DNMT3B

Transcript	Forward Sequence	Reverse Sequence	Probe #
All (common assay)	ATCTCACGGTTCCTGGAGTG	GGTTGCCCCAGAAGTATCG	84
001	TCGTGCAGGCAGTAGGAAAT	TCGTGCAGGCAGTAGGAAAT	83
002	ACAGCAGTCAGGGACAGACATA	TTTGCAGTTTTCCCTGTCATC	60
003	ACAGCAGTCAGGGACAGACATA	TTTGCAGTTTTCCCTGTCATC	60
004	GGCTAATTGCACAGAGCAGTC	GCACCCAGGACAGAGTGG	23
201*			
202*			
203/006*			
204/005	ACAGCAGTCAGGGACAGACATA	TTTGCAGTTTTCCCTGTCATC	60

RPLP0 (‘Housekeeping gene’)

Transcript	Forward Sequence	Reverse Sequence	Probe #
RPLP0	CACTGAGATCAGGGACATGTTG	CTTCACATGGGGCAATGG	74

*not NCBI recognised protein coding transcripts

Green sequences were successfully amplified

DNMT3A protein coding transcripts

Gene: DNMT3A ENSG00000119772

Description	DNA (cytosine-5-)-methyltransferase 3 alpha [Source:HGNC Symbol;Acc:2978]
Location	Chromosome 2: 25,455,845-25,565,459 reverse strand.
INSDC coordinates	chromosome:GRCh37:CM000664.1:25455845:25565459:1
About this gene	This gene has 15 transcripts (splice variants) and is associated with 1 phenotype.
Transcripts	Hide transcript table

Show All entries | Show/hide columns (1 hidden) | Filter

Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
DNMT3A-001	ENST00000264709	4380	912aa	Protein coding	CCDS33157	Q6PJ37 Q8WVA9 Q9Y6K1	NM_175629 NP_783328	GENCODE basic
DNMT3A-011	ENST00000321117	4279	912aa	Protein coding	CCDS33157	Q6PJ37 Q8WVA9 Q9Y6K1	NM_022552 NP_072046	GENCODE basic
DNMT3A-002	ENST00000380746	3589	723aa	Protein coding	CCDS1718	Q6PJ37 Q8WVA9 Q9Y6K1	NM_153759 NP_715640	GENCODE basic
DNMT3A-012	ENST00000406659	1775	166aa	Protein coding	CCDS46232	Q9Y6K1	NM_175630 NP_783329	GENCODE basic
DNMT3A-013	ENST00000402667	2300	689aa	Protein coding	-	Q6PJ37 Q8WVA9 Q9Y6K1	-	GENCODE basic
DNMT3A-010	ENST00000380756	4477	781aa	Nonsense mediated decay	-	F8WE91 Q59HC6	-	
DNMT3A-008	ENST00000474887	847	No protein	Processed transcript	-	-	-	
DNMT3A-017	ENST00000496570	656	No protein	Processed transcript	-	-	-	
DNMT3A-003	ENST00000470983	589	No protein	Processed transcript	-	-	-	
DNMT3A-006	ENST00000461228	581	No protein	Processed transcript	-	-	-	
DNMT3A-015	ENST00000482935	519	No protein	Processed transcript	-	-	-	
DNMT3A-009	ENST00000491288	472	No protein	Processed transcript	-	-	-	
DNMT3A-004	ENST00000474807	840	No protein	Retained intron	-	-	-	
DNMT3A-007	ENST00000466601	812	No protein	Retained intron	-	-	-	
DNMT3A-005	ENST00000484184	619	No protein	Retained intron	-	-	-	

DNMT3B protein coding transcripts

Gene: DNMT3B ENSG00000088305

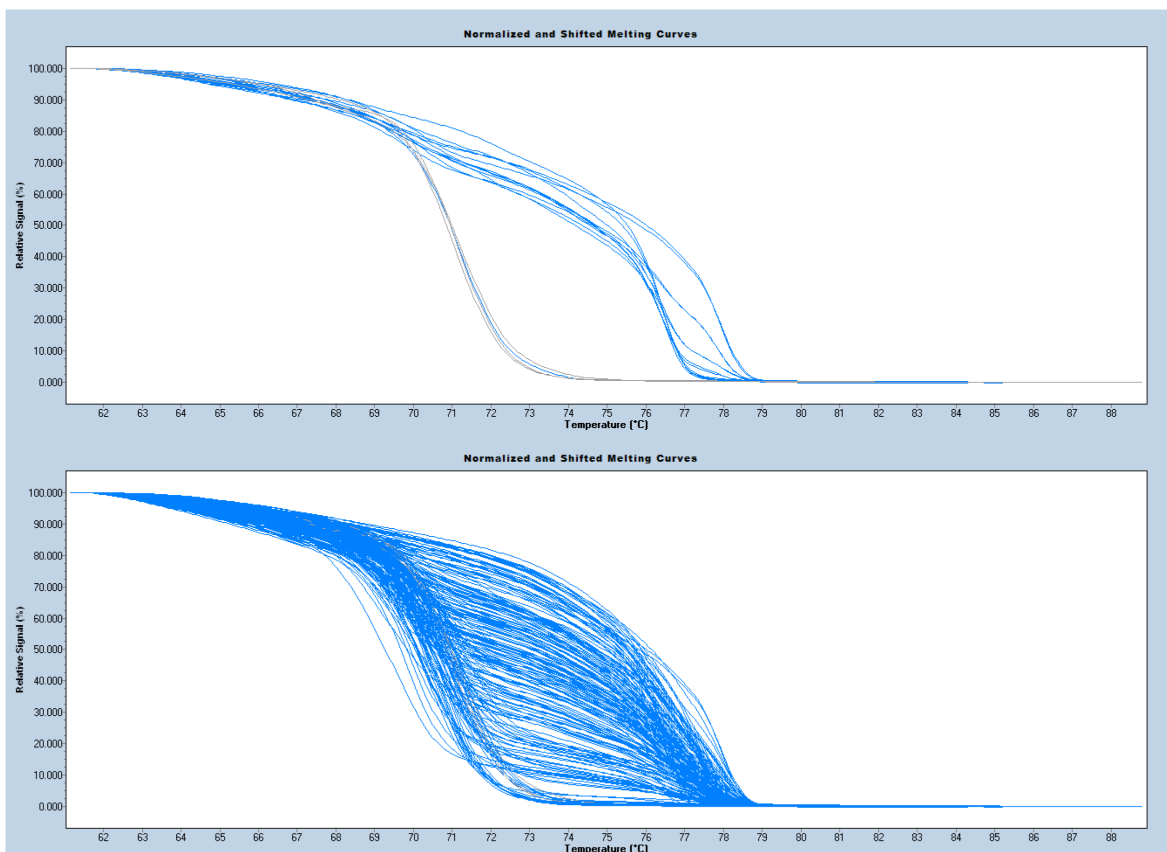
Description	DNA (cytosine-5-)-methyltransferase 3 beta [Source:HGNC Symbol;Acc:2979]
Location	Chromosome 20: 31,350,191-31,397,162 forward strand.
INSDC coordinates	chromosome:GRCh37:CM000682.1:31350191:31397162:1
About this gene	This gene has 8 transcripts (splice variants) and is associated with 3 phenotypes.
Transcripts	Hide transcript table

Show/hide columns (1 hidden) | Filter

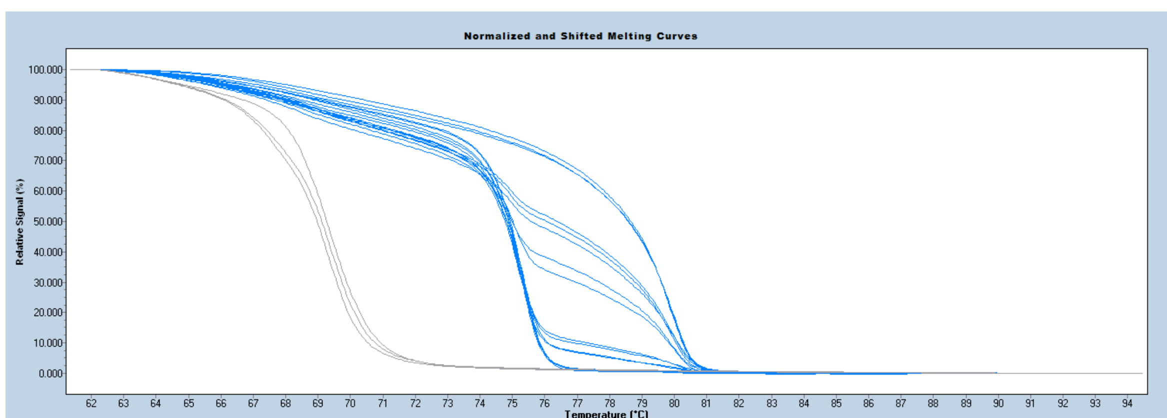
Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
DNMT3B-001	ENST00000328111	4336	853aa	Protein coding	CCDS13205	Q9UBC3	NM_006892 NP_008823	GENCODE basic
DNMT3B-004	ENST00000201963	4255	845aa	Protein coding	CCDS13204	Q9UBC3	NM_175850 NP_787046	GENCODE basic
DNMT3B-002	ENST00000353855	4237	833aa	Protein coding	CCDS13206	Q9UBC3	NM_175848 NP_787044	GENCODE basic
DNMT3B-003	ENST00000348286	4048	770aa	Protein coding	CCDS13207	Q9UBC3	NM_001207055 NM_175849 NP_001193984 NP_787045	GENCODE basic
DNMT3B-006	ENST00000443239	2674	728aa	Protein coding	CCDS56183	Q9UBC3	-	GENCODE basic
DNMT3B-005	ENST00000456297	2315	694aa	Protein coding	CCDS56184	Q9UBC3	NM_001207056 NP_001193985	GENCODE basic
DNMT3B-201	ENST00000344505	3932	792aa	Protein coding	-	Q9UBC3	-	GENCODE basic
DNMT3B-202	ENST00000375623	1709	332aa	Protein coding	-	F8W7Y4	-	GENCODE basic

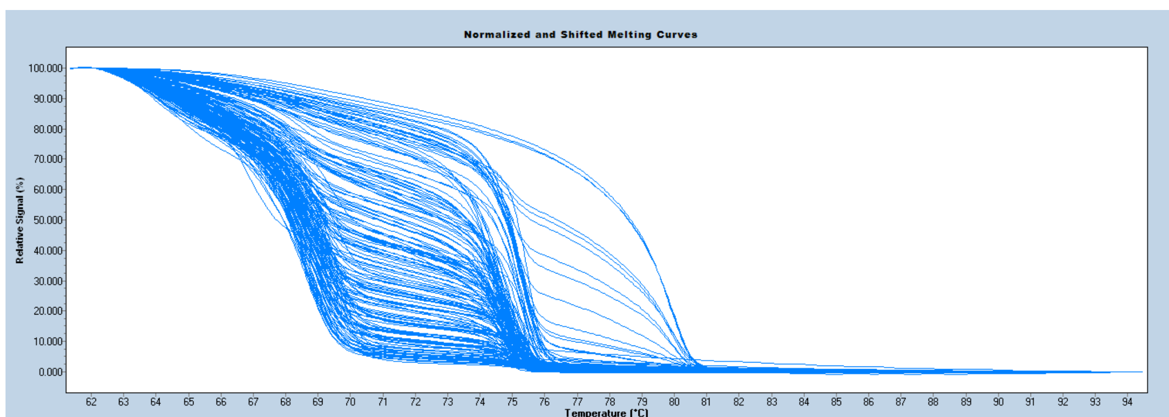
Appendix K – Normalised and Temperature Shifted Melt Curves

Normalised and temperature shifted curves for *IL6* standards (top) and with all participant samples (bottom).

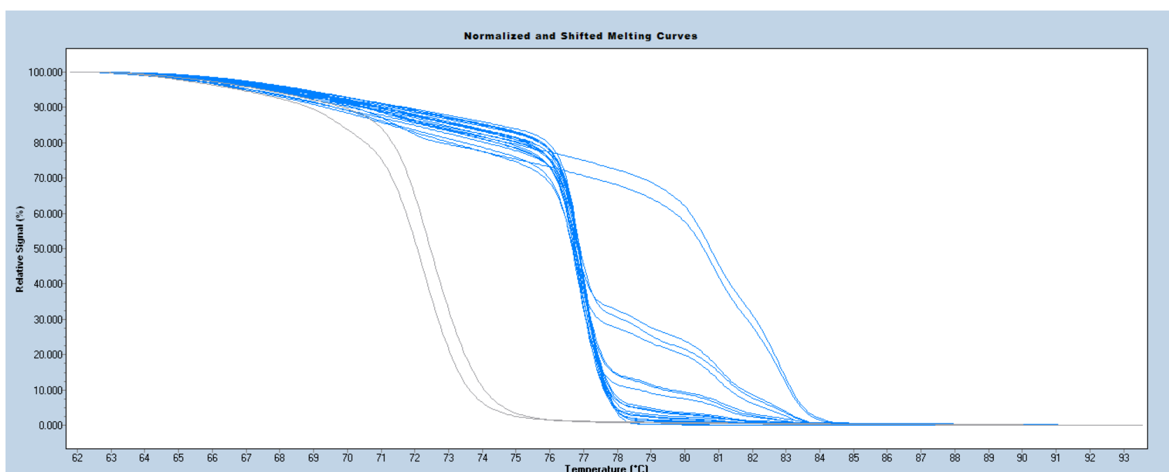


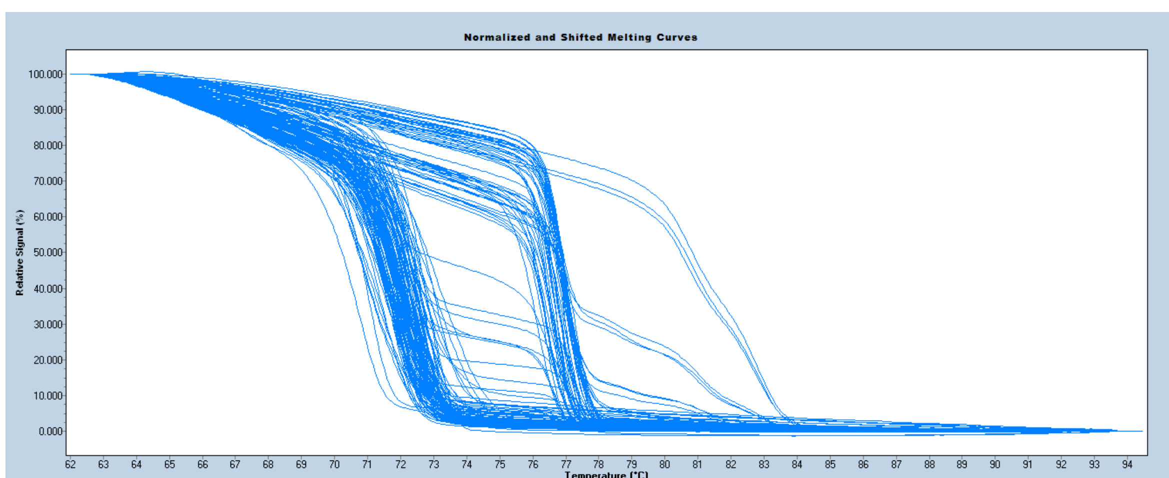
Normalised and temperature shifted curves for *NFκB2* standards (top) and with all participant samples (bottom).





Normalised and temperature shifted curves for *ASC* standards (top) and with all participant samples (bottom).





Appendix L – Figure 8.1 References

1. *Ljunghall et al., 1984; Salveson et al., 1994; Vora et al., 1983*
2. *Hammond et al., 2007; Yamaguchi et al., 1998; Feske, 2007*
3. *Bornefalk et al., 1997*
4. *Hammond et al., 2007; Yamaguchi et al., 1998*
5. *Banzet et al., 2013; Nielsen et al., 2014; Sawada et al., 2013*
6. *Rushworth et al., 2012*
7. *Masumoto et al., 2003*
8. *Özören et al., 2006*
9. *Albrecht et al., 2007; Haseeb et al., 2013; Osborn et al., 1989*
10. *Cogswell et al., 1994; Fujisawa et al., 2011*
11. *Wang et al., 2003*
12. *Shimizu et al., 1990; Son et al., 2008*
13. *Dhar et al., 2013; Osborn et al., 1989*
14. *Collart et al., 1990; Shakhov et al., 1990*
15. *Ke et al., 2007; Ulivi et al., 2008*
16. *Section 1.5.2*
17. *Section 1.5.1/Braconi et al., 2010 (via miRNAs)*
18. *Section 1.5.2*
19. *Greenhough et al., 2009*
20. *Section 1.5.3*
21. *Data presented in this thesis*