Exercise and inflammation-related epigenetic modifications: focus on DNA methylation

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Abstract

Epigenetics is the study of mitotically or meiotically heritable phenotypes that occur as a result of modifications to DNA, thereby regulating gene expression independently of changes in base sequence due to manipulation of the chromatin structure. These modifications occur through a variety of mechanisms, such as DNA methylation, post-translational histone modifications, and non-coding RNAs, and can cause transcriptional suppression or activation depending on the location within the gene. Environmental stimuli, such as diet and exercise, are thought to be able to regulate these mechanisms, with inflammation as a probable contributory factor. Research into these areas is still in its infancy however.
This review will focus on DNA methylation in the context of inflammation (both pro- and anti-inflammatory processes) and exercise. The complexity and relative shortcomings of some existing techniques for studying epigenetics will be highlighted, and recommendations for future study approaches made.

Keywords: DNA methyltransferase, NLRP3, stress, glucocorticoid, physical activity.

1.0 - INTRODUCTION

Although epigenetics is an emerging area of research within sport and exercise sciences, it has been of interest to the wider scientific community for many decades. The word 'epigenetics' was first coined by Conrad Waddington in 1942, later defined as 'a branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' (90). This broad description has since been refined and is generally accepted nowadays as meaning, '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' (98).
Epigenetics seems to go against the traditional principles of genetics where early 20th century data supports the Darwinian theory that genes are the basis of phenotype, and any change in phenotype is due to alterations in DNA sequence. A competing but generally discredited hypothesis to Darwin's concept of evolution was the one proposed by Jean-Baptiste Lamark in 1809. The Lamarkian theory of heritability of acquired characteristics suggests that traits acquired during a lifetime can be passed on to future generations. This theory was generally abandoned in biology and replaced by the classical Mendelian laws of inheritance, namely, the law of segregation, the law of independent assortment, and the law of dominance. Indeed, it was widely accepted that the only way for traits to be passed on through generations was through the inheritance of genes and that the environment could not influence them. Lamark's theory, that environment plays a role in inherited phenotype, is now being recredited by the scientific community.
In light of a greater current understanding of epigenetic change, and the recent evidence indicating a role for the epigenome in inheritance and development, an appreciation that the genome and epigenome work 'in concert' is of paramount importance to future research. By acknowledging the combined influence of both genetic and epigenetic factors, significant progress is being made on the molecular understanding of the pathogenesis of many disease states and resultant therapeutic interventions. In future, due to the apparent dynamic nature of epigenetic changes, it may be possible to prescribe lifestyle interventions to prevent the accumulation of aberrant modifications to the epigenome that are associated with disease and ageing. Research into the impact of environmental stimuli, such as diet and exercise, is still in its infancy however. Thus, this area represents a worthwhile and fruitful avenue of investigation for sport and exercise science research.

The following review serves to provide a background understanding of epigenetic mechanisms and in particular the role of DNA methylation in normal functioning and in the pathogenesis of disease. Modifications to methylation in the context of inflammation and exercise will also be discussed.

2.0 - EPIGENETICS: UNDERSTANDING THE EPIGENOME

2.1 - Fundamentals of Epigenetics

DNA consists of nucleotides: a deoxyribose molecule bound to a phosphate group on one side, creating the backbone of DNA, and bound to one of four nitrogenous bases on the opposing side. The double-ringed purine bases Adenine (A) and Guanine (G) pair with the single-ringed pyrimidine bases Thymine (T) and Cytosine (C) (A with T, G with C) (figure 1). Nucleosomes, which consist of ~147 base pairs of double

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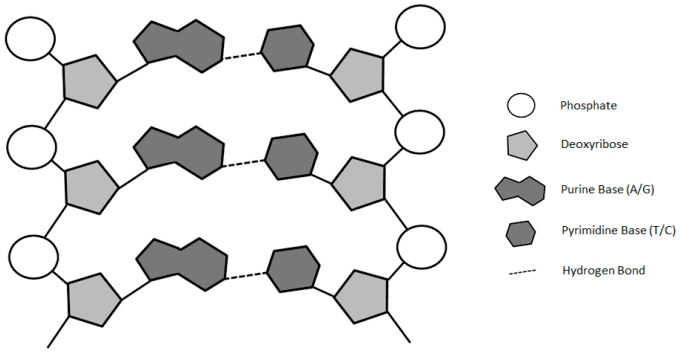

Figure 1 - Schematic of the molecules that make up a short section of DNA.

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 (figure 2). Post-translational modifications to histones are key moderators of gene activity, with acetylation and methylation the best characterised, although ubiquitation, phosphorylation, sumoylation, ADP-ribosylation and citrullination also occur.

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 acetyltransferase (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 formation.

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.

Non-coding RNAs (ncRNA), RNA molecules that are not translated into a protein, can be classified into many subgroups, including, but not limited to, micro RNAs (miRNA), involved in post-transcriptional gene silencing; piwi-interacting RNAs (piRNA), which direct DNA methylation at transposable elements; and long non-coding RNAs (lncRNA), which direct epigenetic machinery such as chromatin remodelling complexes.

There is a complex interplay between histone modifiers, chromatin remodelling complexes, ncRNAs, and DNA methylation, however, for the purpose of this review, only DNA methylation will be discussed further.

2.2 - DNA Methylation

DNA methylation, characterised by the DNA methyltransferase (DNMT) regulated addition of a methyl group to the nucleotide cytosine, creating 5-methylcytosine (5mC), 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 (51). Clusters of CpG dinucleotides are often located at transcription start sites of genes known as promoter regions, and although DNA methylation has also been found to occur at non-CpG sites (33), the process is more commonly reported at the former. The effect of methylation at gene promoter CpG islands is transcriptional silencing of gene expression, 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 (5).

A number of DNMTs regulate the methylation process (figure 3). DNMT1 methylates hemi-methylated DNA, and therefore, has an important role with regards to the maintenance of methylation. 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 (72), as well as during methylation of maturing gametes (35, 81). 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-adenosyl-L-methionine (SAM) (46). 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 (13). All three of the aforementioned DNMTs are essential in mammalian development, as demonstrated by the death of DNMT deficient mice (52, 72). 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 (22). 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 (6).

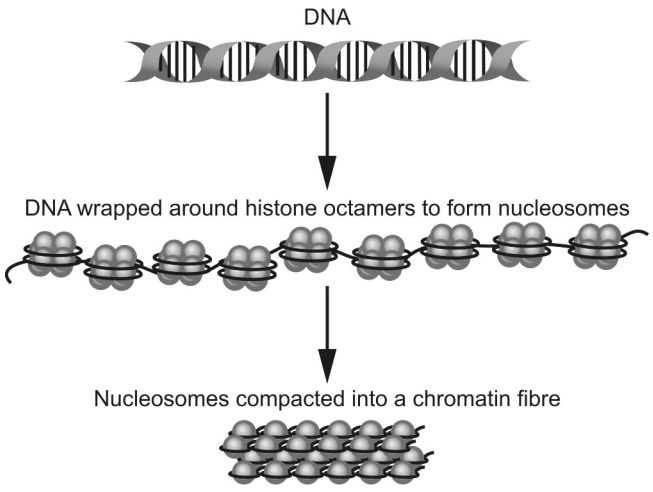

Figure 2 - Structure of a chromatin fibre (image provided courtesy of Abcam Inc. Image copyright©2014 Abcam).

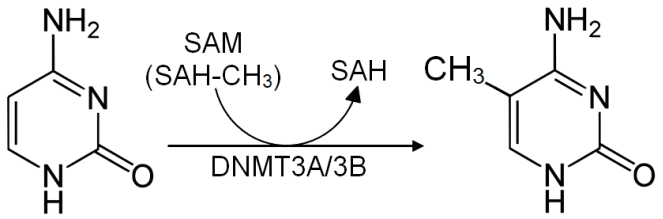

Figure 3 - DNMT regulated transfer of a methyl group (H3C) from the methyl donor S-Adenosyl methionine (SAM), converting cytosine (left) into 5-methylcytosine (right).

Despite sharing structural similarity to the other DNMTs, DNMT2 is located primarily in the cytoplasm, in contrast to DNMT1, DNMT3A and DNMT3B, which are located in the nucleus. Additionally, DNMT2 does not appear to alter genomic methylation, as demonstrated by DNMT2 deficient mouse embryonic stem cells, but rather, methylates aspartic acid transfer RNA ($tRNA^{Asp}$) (32).

As a brief example of the overlap between epigenetic mechanisms, miRNA-143 downregulates DNMT3A mRNA and protein levels in colorectal cancer cell lines (67), while both DNMT3A and DNMT3B have been shown to be direct targets of miRNA-29 in lung cancer samples (24). Similarly, DNMT1 has been verified as a target for miRNA-148a and miRNA-152 (9).

2.3 - 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 5mC. 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. DNA glycosylases, involved in base excision repair of damaged DNA, have been considered to be involved in the demethylation process, however, the identification of 5-hydroxymethylcytosine (5hmC) via the TET1 (ten-eleven translocation enzyme) mediated oxidation of 5mC (85), was of key importance in understanding the molecular mechanisms of active demethylation. Following 5mC oxidation, a number of possible pathways for demethylation have been proposed, including passive dilution of the oxidised base, direct removal of the oxidised 5'-position substituent, and DNA repair-mediated excision of modified nucleotides (50). This section serves as a brief summary of the current understanding of the demethylation process, and the reader is referred to the recent review by Kohli and Zhang (50) for elaboration on the topic.

2.4 - Role of Methyl-CpG Binding Proteins

MBPs play an important role in transcriptional repression and heterochromatin (closed) structure formation (figure 4). Three structural families have been identified; methyl CpG-binding domain (MBD), Zinc Finger, and SET and RING finger-associated domain (SRA). 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 histone deacetylase (HDAC) complex at methylated regions, which results in the repression of chromatin structure (91). There are, however, other mechanisms, including interactions between MeCP2 and histone methyltransferases (HMT) (31). 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 (15). 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 aiding the maintenance of methylation. Thus far, UHRF1 is the only MBP that has been shown to bind 5hmC as well as 5mC (11).

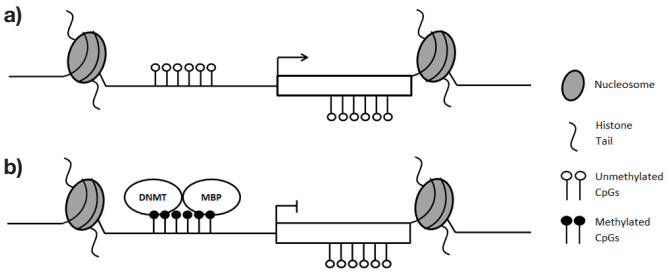

Figure 4 - a) Unmethylated CpG dinucleotides at a gene promoter region. Gene is active; b) DNMT mediated methylation of CpG dinucleotides, followed by MBP recruitment which causes chromatin remodelling and blocking of transcription factors, resulting in transcriptional suppression.

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. The syndrome is caused by germline mutations in MeCP2 and is commonly mistaken for autism during the early stages of onset, while common symptoms include microcephaly, chorea, ataxia, apraxia, and seizures. 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 (19). 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.

Up to now, the discussion of epigenetic modification, such as changes in DNA methylation status, have been focused on findings reporting disease or condition-specific epigenetic changes which are directly associated with causing a specific illness, such as the recent review linking immunity, cancer and epigenetics in the context of inflammatory bowel disease (17). However, literature focused on non-disease related epigenetic changes with relevance to inflammatory processes, is relatively lacking.To add complexity to the interpretation of epigenetic data, more general epigenetic changes that are seemingly unrelated to a particular disease, also occur. These

general changes may alter the cellular environment in such a manner as to predispose an individual to a number of diseases. One example of such a general change which may increase susceptibility to various chronic diseases, and one that is very relevant to the exercise arena, is inflammation, which will be discussed in this context in the next section.

3.0 - INFLAMMATION: INTERLINKED ROLES OF THE INFLAMMASOME AND GLUCOCORTICOIDS IN MODIFYING DNA METHYLATION STATUS?

Sterile inflammation is increasingly named as a secondary aetiological factor in modern lifestyle related diseases, such as cardiovascular disease (70), diabetes (73) and depression (38). Chronic stress is a further aetiological role player, since chronic activation of the glucocorticoid system, and subsequent insensitivity to glucocorticoids is known to contribute to low grade inflammation. Furthermore, obesity-related chronic low grade inflammation is also implicated on an epigenetic level in the development of some forms of cancer, such as colorectal cancer (56), further highlighting the prominence of a chronic inflammatory condition as an adverse health factor. Interestingly, for most lifestyle-related diseases such as the aforementioned, moderate exercise, a known anti-inflammatory modality, is prescribed as a preventative and/or complementary treatment. However, the plasticity of exercise-induced changes, and thus its longer-term impact on inflammation and/or glucocorticoid resistance in the context of the development of these pathologies, could be largely dependent on epigenetic modification. In this section, following a brief background on the (non-epigenetic) inflammasome and related immunology, relevant literature available on the epigenetic changes associated with inflammation and glucocorticoid function will be discussed, followed by the reported modulatory effects of exercise.

3.1 - Linking Peripheral Inflammatory Markers to the Inflammasome

3.1.1 - Sterile Inflammation and Innate Immunity

Sterile inflammation is commonly known as the response to either psychological or physical stressors that evoke an innate immune response, in the absence of pathogenic stimuli (23, 59). The exact mechanism of activation remains unclear, though several signals that trigger the immune response have been identified, such as catecholamines, glucocorticoids, intestinal microbiota, as well as molecular signals from host tissue.

Among these signals recognised by the innate immune system are danger-associated molecular patterns (DAMPs), which account for the initiation of an inflammatory response in the absence of microbial stimuli (26). In response to stressors, the host tissue releases these danger signals in response to a local and/or systemic challenge. DAMPs, like pathogen-associated molecular patterns (PAMPs), share several characteristics: they are host-derived proteins, endogenous within cells, and go undetected by the immune system. Several of these DAMPs have been identified in recent years, with many being endogenous molecules that act as alarm signals when released extracellularly, for example, high mobility group 1 (HMG1), ATP, uric acid, glucose and heat shock proteins (23, 59).

Regardless of how the inflammatory branch of the innate immune system is activated, it inevitably results in the appearance of pro-inflammatory markers that are commonly measured and reported in the exercise science literature.

3.1.2 - Importance of IL-1β and the NLRP3 Inflammasome

IL-1β is one of the most important and most potent inflammatory mediators. In its active form, this inflammatory cytokine is primarily released from myeloid cells such as monocytes, macrophages and dendritic cells, but is also readily secreted by most other tissues on stimulation. IL-1β triggers the acute phase response, characterised by the release of C-reactive protein and amyloid β from the liver, the release of inflammatory cytokines such as IL-6 and TNF-α, and secretion of adrenocorticotropic hormone. It also evokes the symptoms fever and hypotension within the host via a plethora of chemical mediators.

Upon stimulus, IL-1β is produced in its inactive 35kDa form. Proteolytic cleavage with caspase-1 results in the generation of the mature 17kDa protein. The process of activation of caspase-1 is essential for IL-1β maturation, and is tightly regulated by multi-protein complexes, known as inflammasomes. To date, several inflammasomes have been identified, namely, NLRP1, 2, 3, 6, NLRC4 and AIM-2. The NLRP3 inflammasome, activation of which regulates IL-1 β and IL-18 in obesity (89), is the most well studied and will be the focus of this section of the review.

3.1.3 - NLRP Inflammasome Structure

The NLRP3 inflammasome (also known as cryopyrin and NALP3) is a multiprotein complex expressed in myeloid cells. This structure consists of a central nucleotide binding

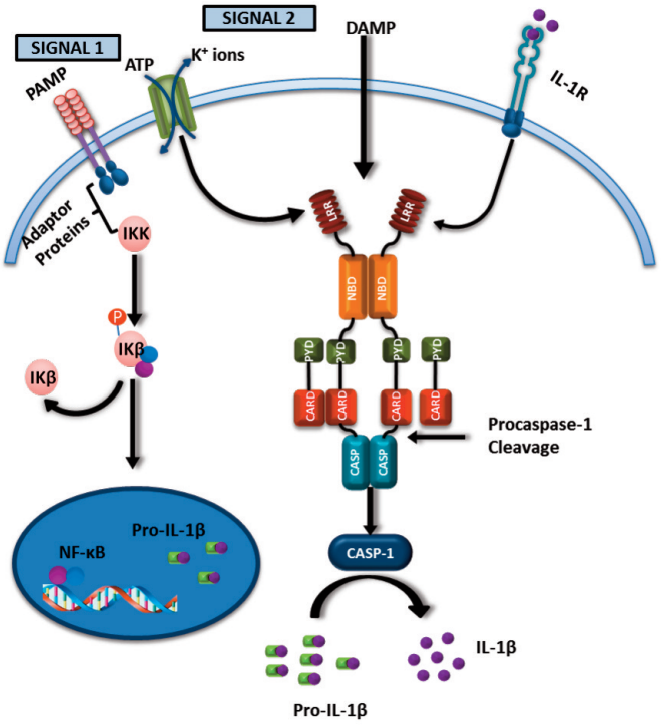

Figure 5 - Schematic illustration of NLRP3 inflammasome activation and subsequent intracellular signalling that produces a pro-inflammatory outcome.

domain (NBD/NACHT/NOD), together with a C-terminal LRR domain. This structure lacks a caspase recruitment domain (CARD), and requires an adaptor molecule (apoptosis-associated speck-like protein containing a CARD (ASC)) to recruit a procaspase-1. NLRP3 interacts with ASC via PYD homophilic dimerization. Similarly, NLRP3 interacts with CARD8 (also known as CARDINAL), to achieve activation of caspase-1. The NLRP3 activation cycle is presented schematically in figure 5.

NLRP3 activation markers are divided into two categories, namely sterile, which include host and environmental stimuli (extracellular ATP, hyaluronic acid, fibrillar amyloid β, silica, asbestos, uric acid), and non-sterile, pathogen-associated activators, which are PAMPs from bacteria, fungi, virus and protozoa.

NLRP3 activation can be triggered by direct PAMPs/DAMPs binding to pattern recognition receptors (PRR) (signal 1), or with incorporation of an addition external ATP (signal 2). The stimulation of PRRs by PAMPs leads to the activation of adaptor proteins, such as MyD88 that activate IKK complexes. These complexes phosphorylate IκB proteins, and subsequently results in ubiquitination and degradation by the proteasome. This frees the p50/p65 heterodimer of the NF-κB, which enters the nucleus and activates gene transcription. Among the cytokines release, pro-IL-1β is expressed.

External ATP acts as a second signal of NLRP3 inflammasome activation, resulting in the efflux of K^+ ions from the P2X7 receptor. Endogenous DAMPs are also able to trigger inflammasome activation. Upon NLRP3 activation, the NLRP3 oligomerizes and results in PYD domain clustering, which leads to the homotypic interaction with the PYD and CARD domains of the ASC adaptors. The CARD domains of the ASC, in turn, react with the CARD of pro-caspase-1, which allows for auto-cleaving and the construction of an active caspase-1 p10/20 tetramer. Caspase-1 is able to produce active IL-1β through its cleave of the pro-IL-1β molecules.

3.2 - Interdependency of Inflammasome and Glucocorticoids: Relative Lack of Studies on Epigenetic Involvement

In the context of non-sterile inflammation, lipopolysaccharide (LPS) infection is known to increase pro-inflammatory cytokine concentrations (IL-1β, IL-6, TNF-α) and to activate the HPA-axis. In a model of maternal infection, LPS infection of mothers during late gestation (day 17) was associated with higher stress responsiveness (higher corticosterone) and anxiety behaviour ('elevated plus maze' rodent model of anxiety) in offspring, both in adolescence (day 40) and adulthood (day 80) (23). Although the mechanisms by which these effects were facilitated were not investigated, it suggests transgenerational transfer of effects resulting from inflammation, thus potentially, epigenetic modulation.

Furthermore, in the context of severe trauma, known to result in a glucocorticoid response, acute stress in the form of 100 tail shocks in rats has been shown to activate the inflammasome to increase circulating inflammatory cytokine (IL-1β, IL-18, IL-6, IL-10 and MCP-1) and danger associated molecular pattern (DAMP – hsp72 and uric acid) levels, in a caspase-1 dependent manner (59). In this study, use of a caspase-1 inhibitor attenuated the stress-induced pro-inflammatory response (IL-1β, IL-6 and IL-18) both in the circulating and tissue compartments. Furthermore, the DAMPs assessed were implicated in the caspase-1 activation seen after stress exposure. From this study it is clear that a connection exists between the stress response and inflammation. Despite this, we could not find any studies jointly reporting on both inflammation and glucocorticoid epigenetic changes in the absence of specific pathology such as cancer. In our opinion, given the proven links between these responses reported more downstream, as shown above, this omission is an important gap in the literature that should be addressed. Therefore, while reviewing epigenetic changes impacting on inflammation here, we have included what is known about epigenetic modification in the context of the glucocorticoid response, to encourage inclusion of these parameters in future studies for a more all-encompassing approach.

3.2.1 - Information from Stress Studies

In considering stress and glucocorticoid-related epigenetic modification as an "additive factor" determining the susceptibility to inflammation-induced epigenetic changes, it is important to consider both acute and chronic changes in the glucocorticoid system. Ever since the first maternal separation rodent study by Levine in the 1950s (53), stress as an early life environmental factor has been known to have long-term deleterious effects on stress-susceptibility into adulthood. Similarly, rodents raised by non-caring mothers, who neglected to lick and groom pups during the first week after birth, resulted in decreased resistance to stress. Interestingly, this effect could be reversed by cross-fostering pups with more caring mothers directly after birth (28), suggesting that individual differences in stress reactivity were not genetically inherited, but likely occur via nongenomic transmission in the early developmental phase. Although the critical site for glucocorticoid receptor (GR) regulation remains to be identified, increased nerve growth factor-induced protein A (NGFI-A), also known as early growth response protein-1 (EGR-1), expression has been linked to up-regulation of GR in the hippocampus (63). However, since the increased NGFI-A expression seen in offspring of caring mothers does not persist into adulthood, while the beneficial effect on stress reactivity does, data again points toward epigenetic modification.

Indeed, more recent epigenetic studies confirmed this. Rodent studies showed that the 5' CpG dinucleotide of the NGFI-A consensus sequence within the exon I_7 GR promoter is always methylated in offspring of non-caring mothers, while in offspring of caring mothers, it is rarely methylated. After cross-fostering offspring to caring mothers, this methylation was reversed, suggesting site-specific DNA methylation silencing of the GR promoter is reversible by environmental factors, in this case, maternal care (93). Interestingly, in this study, modification of DNA methylation was shown to occur in cytosines at very specific sites, since, for example, the neighbouring 3' CpG dinucleotide of the AP-1 consensus sequence within the exon I_7 GR promoter was not affected. Thus, it appears likely that epigenetic modification of DNA methylation by any particular intervention is a highly specific, targeted response. Of specific interest was the fact that these patterns in methylation were not present at birth, but developed within the first 6 days of life (12), ruling out genetic inheritance of stress reactivity.

Rather, postnatal maternal conduct seems to play a huge role in the epigenetic outcome of offspring in the context of glucocorticoid receptor expression and stress reactivity. An important message here is that even a relatively acute stressor, in this case only 6 to 7 days, may result in epigenetic modification that persists chronically. Thus, relatively acute stressors may alter chronic glucocorticoid sensitivity and thus more chronically predispose an individual to pro-inflammatory epigenetic modification.

The studies in rodents as described above may give the impression that dynamic DNA methylation and demethylation occurs early in life only. However, treatment of adult rodents with the HDAC inhibitor trichostatin A (TSA) for 4 days was reported to significantly increase histone acetylation at the exon I_7 site, which increased NGFI-A protein binding and resulted in demethylation of the CpG dinucleotide of the NGFI-A consensus sequence within the exon I_7 GR promoter (93). This suggested that the DNA methylation status in fully differentiated cells can be modified, which has far-reaching therapeutic implications.

Taken together, in the context of stress, DNA methylation patterns seem to be largely dependent on environmental and maternal influence early in life. However, pharmacological intervention seems to be an option, at least theoretically, for modification of DNA methylation status in adulthood to reverse this "environmental programming". The extent to which environmental changes may influence this epigenetic programming is still uncertain.

Although the link between chronic stress and inflammation is well established in non-disease models, for example, in humans exposed to the chronic stress of maltreatment during childhood (62), literature on epigenetic links in this context seems to be lacking. Also, in disease conditions linked to chronic stress, pro-inflammatory changes have been reported; in the context of major depressive disorder, increased NLRP3 inflammasome activation was very recently found (3). In this study, increased gene expression of NLRP3 and caspase-1 was discovered in mononuclear blood cells (PBMCs), and was associated with increased serum IL-1β and IL-18 levels, both of which correlated with depression scores, according to the Beck Depression Inventory questionnaire. Similarly, chronic glucocorticoid treatment in rats was shown to increase gene expression of NLRP3, Iba-1, MHCII and NF-κBIα in the rat hippocampus (29). Thus, it is clear that chronic stress has a pro-inflammatory outcome that is associated with the NLRP3 inflammasome. Frustratingly however, direct proof of epigenetic involvement is once again, not available. This point was highlighted in a recent review (44) which stated that the epigenetic mechanisms for the upregulation of the NLRP3 inflammasome have not been elucidated. It is uncertain, therefore, whether only the NLRP3 gene is modified epigenetically, or whether glucocorticoid-associated genes, such as NGFI-A, are also implicated. The targets of stress-induced epigenetic modifications with inflammatory outcomes have huge therapeutic implications, so that the relative absence of epigenetic studies linking stress and inflammation highlights the need for a multidisciplinary approach in epigenetic studies.

Since the epigenetic mechanisms resulting in activation or inactivation of specific pro-inflammatory processes, such as the NLRP3 inflammasome, have not been elucidated, especially not in the absence of pre-existing disease, we will briefly review what is known on epigenetic modulation of these systems in the context of different diseases with an inflammatory component, trying to tease out the commonly reported parameters associated with inflammation and not with the primary disease itself, before moving on to a discussion of epigenetic modulation by physical activity (PA) and exercise.

3.3 - Inflammation and DNA Methylation in the Context of Inflammation-associated Conditions

While DNA methylation is an essential component of normal development and transcriptional regulation, aberrant patterns of DNA methylation are associated with a number of inflammatory diseases and conditions. However, in terms of epigenetic modification and inflammation, the causal directionality remains questionable. This is briefly discussed here, in the context of a variety of inflammatory models.

Obesity is well-known to contribute to a chronic low-grade inflammation via increased secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β from macrophages infiltrating adipose tissue (73). In this context, high-fat diet feeding in two subsequent generations of mice has recently been reported to result in DNA hypomethylation of inflammation-associated genes in adipose tissue of third generation mice (18). One of the most notable findings in this study was an upregulation of NLRC4, a critical component in the formation of the inflammasome which plays an important role in obesity-related inflammation, in both first and second generation offspring (90). Another significant upregulation was that of the toll-like receptors (TLRs), and specifically TLR4, which facilitates obesity-induced inflammation by inhibition of PCK1. Notably, the study also reported hypomethylation of inflammation-associated promoters of the genes TLR1, TLR2 and Lat. This data is in accordance with other inflammatory models. For example, an inverse correlation between DNA methylation of TLR4 and the inflammatory response to LPS stimulation in intestinal epithelial cells has been reported (86), while DNA methylation of TLR2 in circulation was also inversely associated with the C-reactive protein, ICAM-1 and VCAM-1 responses to air pollution (8). Furthermore, in the context of cystic fibrosis (CF), a disease characterised by chronic inflammation and recurring infections of the pulmonary tract, upregulation of TLR2 in a CF bronchial epithelial cell line was due to DNA hypomethylation (83). Finally, hypomethylation of a number of gene promoters has been reported in synovial fluid of rheumatoid arthritis (RA) patients. These included CXCL12 (47), as well as CHI3L1, CASP1, STAT3, MAP3K5, MEFV, and WISP3 (66), many of which are associated with inflammation. Even in circulation this effect was evident, with hypomethylation of a single CpG motif of the IL-6 gene reported in PBMCs of RA patients (68). Augmented expression of these genes could contribute to RA due to their roles in the activation and differentiation of various immune cells and pro-inflammatory cytokines.

Thus, in lieu of epigenetic data from non-disease models of inflammation, by using obesity as a disease-free model of inflammation, and comparing results reported in this model to that of inflammatory diseases, we have been able to identify epigenetic changes associated with clinical symptoms of inflammation. The available evidence appears to indicate that aberrant DNA methylation contributes to inflammation

through hypomethylation and subsequent upregulation of inflammatory gene expression.

3.3.1 - Are Athletes at Risk of Aberrant Inflammation-induced Epigenetic Modifications?

Up to now, this section has focused on DNA methylation changes as a causal factor in the development of chronic inflammation. However, we should also address the question of whether this communication is bidirectional, i.e. can chronic inflammation in the absence of pre-existing disease also lead to other epigenetic modifications? 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 colorectal cancer manifests with high levels of IL-6 (4), while DNMT1 is also overexpressed in this condition (27). *In vitro* studies have shown that IL-6 stimulation of human cancer cell lines (HCT116/K562) resulted in elevated expression and activity of DNMT1 (27, 42). This augmentation of DNMT1 could occur through PI3K activation of AKT, and subsequent AKT dependent phosphorylation of the nuclear localisation signal of DNMT1, allowing nuclear translocation and subsequent binding of DNA (39). IL-6 has also been implicated in the maintenance of promoter methylation in cholangiocarcinoma (94) and multiple myeloma cells (41), 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) cells (55). 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 (104). Furthermore, IL-6 induced DNMT1 mRNA and protein expression correlated with SOCS3 promoter methylation via STAT3 signalling in human colorectal cancer cell cultures (54).

Similar to the IL-6-stimulation studies, IL-1β stimulation of fibroblast-like synoviocytes isolated from RA patients subsequently decreased expression of DNMT1, whereas both IL-1β and TNFα decreased levels of DNMT3A (65). TNFα-induced inhibition of Notch-1, a transmembrane protein, has been attributed to Ezh2 (histone methyltransferase) and DNMT3B recruitment via NF-κB, resulting in hypermethylation in mouse myoblast cells (1).

Interestingly, a recent study (79 - discussed in more detail in the next section) also provides support for the link between transient exercise-induced increases in plasma IL-6 and methylation status of several genes related to immune function, in a sample of healthy, trained men.

Given these findings, it is possible that the acute inflammatory state associated with intense exercise (25) could drive alterations in the epigenome, possibly predisposing athletes to disease. Despite the fact that many of the aforementioned studies were conducted *in vitro* using various cancer cell lines, the findings of Robson-Ansley et al (79) corroborate the notion that exercise-induced inflammation may be related to modifications to DNA methylation in a non-diseased population such as athletes.

4.0 - EXERCISE AND EPIGENETIC MODIFICATION

In contrast to the bleak picture painted above in terms of the potential aberrant epigenetic effects of excessive exercise, chronic moderate exercise is commonly accepted to decrease levels of inflammatory biomarkers (30, 84) and reduce the risk of developing many major non-communicable diseases. Data linking exercise and altered DNA methylation suggests that there may be a possible epigenetic mechanism with regards to these protective effects. The following section is a review of all known data linking PA and exercise to DNA methylation (see table 1).

4.1 – 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 methylation, i.e. changes in methylation across the genome. The association between PA, measured by accelerometry over four days, and global methylation in LINE-1, a marker shown to correlate well with other measures of global methylation (97), has been investigated (102). Those who performed approximately 30 minutes of PA per day, as measured by a portable accelerometer, when compared to those who performed less than 10 minutes, had a significantly greater level of global DNA methylation. However, following multivariate adjustment for age, gender, smoking status, ethnicity and body mass index (BMI), the association was no longer statistically significant, and thus, this data is more difficult to interpret.

More recently, White et al (96) 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 LINE-1 methylation than those below the median. A trend was also reported for women that performed above the median for one or two time periods. Similarly, in an elderly population, Luttropp et al (57) divided 509 individuals aged 70 years and over into four discrete groups based on the amount of light and heavy PA they performed. 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, LDL and HDL cholesterol, serum triglycerides, smoking status, and BMI.

However, in contrast with the above results which seem to suggest that PA is associated with hypermethylation of DNA across board, results from the Commuting Mode and Inflammatory Response Study did not support this association, as PA was not correlated with global (LINE-1) methylation. Interestingly, levels of IL-6 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 (103). 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 (82). Maintenance of increased PAEE of 500 kcal or more per week, resulted in significant hypermethylation of the

TNF gene, while the IL-10 gene was significantly hypomethylated in those who increased their PAEE by 500 kcal per week, compared with those who decreased their PAEE by 500 kcal per week. Given the pro-inflammatory role of TNFα, and the anti-inflammatory role of IL-10, these PAEE-induced modifications are a favourable outcome. These two studies differed drastically from a methodological standpoint; the former (104) measured PA through the use of a questionnaire that selected 26 specific activities performed over the previous year, in contrast to the latter study (82) which involved participants recalling any physical activities over the previous eight years. Furthermore, Shaw et al's (82) study utilised a much more defined elderly age group in comparison with the Zhang et al's (104) 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 (78), six CpG sites showed differential methylation between the groups, with the more experienced group demonstrating a slowing of the usual age-related pattern of hypo- or demethylation change. This result, as well as the fact that age-related hypomethylation has been reported in PBMC samples (1), 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.

From these studies, 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 mentioned above, the overall inconsistency in results is likely a product of the utilisation of global methylation as an outcome measure, as this does not reflect changes in DNA methylation at the gene-specific level. For example, particular 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 de- or hypomethylation of particular genes is an undesired outcome, hypomethylation, and thus transcription, of tumour suppressor genes is highly desired in the context of cancer. It is thus clear that neither hyper-, nor hypomethylation is desired across the board for all genes, illustrating that interpretation of a "crude" assessment such as global methylation has limited value. This highlights the need to investigate gene and CpG sequence-specific changes. This point has already been illustrated by the gene-specific epigenetic changes reported in other disciplines, as also described in the overview of the stress-related literature in section 3.2.1.

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

In addition to gene-specific studies in the context of inflammation, several studies have attempted to elucidate the epigenetic effects of PA related to disease-specific genetic loci. For example, Coyle et al (16) 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 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, a tumour-suppressor gene, in gastric carcinoma patients (100), suggesting an anti-tumorigenic effect, although no significant associations were reported for the remaining five tumour-related genes (CDX2, BMP-2, 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.

Given the relative complexity of measuring physical activity, which may differ substantially between individuals, in combination with cancer, which again, differs substantially 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 of this intriguing area.

4.3 - Epigenetic Effects of an Acute Exercise Bout

With regard to an acute bout of exercise, global methylation of vastus lateralis skeletal muscle was reported to be reduced in a sample of sedentary young men and women following a VO_{2peak} test on a cycle ergometer. Further analysis demonstrated that hypomethylation occurred at promoter regions of PGC-1α, PDK4 and PPAR-δ immediately post exercise. Consequently, transcription was upregulated, and given the role of these genes in metabolism, this would be regarded to be a health-beneficial outcome. This appears to contradict the hypothesis that an intense acute bout could have deleterious epigenetic effects via inflammatory mechanisms, although in this case, the bout may have simply been too short or not intense enough to elicit a drastic inflammatory response. The fact that the methylation status of muscle-specific transcription factors MEF2A and MyoD1 remained unchanged (7) supports this notion. Another possible explanation is that IL-6 (section 3.3.1) and other pro-inflammatory proteins only regulate the epigenetic machinery involved in hypermethylation, whereas demethylation, as in the context of this study, is a consequence of other regulatory pathways.

Bisulfite sequencing, a technique used for validation of DNA methylation, demonstrated that non-CpG sites (CpA, CpT, CpC) comprised the majority of modified cytosines in this study. It has been suggested that oxidation of the cytosine's methyl group could provide a possible mechanism as to how an acute exercise bout could cause demethylation. However, due to poor specificity of the bisulfite technique with regard to distinguishing between methylated and hydroxymethylated cytosines (43) this mechanism has not yet been clarified. TET-assisted bisulfite sequencing (TAB-seq) on the other hand, is able to quantify 5mC and 5hmC independently from one

another (99), and thus, may be a more appropriate method when investigating changes in demethylation.

A recent study (21) investigated the importance of exercise intensity on epigenetic changes in terms of mitochondrial biogenesis. Healthy male subjects performed interval cycling at 73, 100 or 133% of peak power output (PPO) and post-exercise changes in gene expression of PGC-1α and its regulators were assessed in skeletal muscle biopsies. Cycling at 100% of PPO was reported to increase PGC-1α mRNA more than cycling at 73% PPO, but supramaximal exercise seemed to blunt this response, so that a lower increase in levels of PGC-1α mRNA was seen when compared to both 100% and 73% PPO. Interestingly, increases in the mRNA levels of the regulators Sirt-1, PDK4 and RIP140 occurred in a manner independent of exercise intensity and muscle activation. This upregulation of PGC-1α is regulated by HDACs, one of the ways in which adaptation to exercise is facilitated (74). Although these results aren't directly related to DNA methylation, a recent broad review on epigenetic modulation by exercise (71) pointed out that this mechanism may suggest a way by which the hypermethylated status of PGC-1α in diabetic patients (6) could be modified. A relative lack of literature dealing with inflammasome epigenetics indicates a huge area for future research focus, especially since the PGC-1α results above suggest that at least some of the adaptive epigenetic changes seen after exercise may, in fact, translate to a more permanent and prolonged beneficial outcome.

The effect of acute exercise on cells of the immune system has recently been investigated (79). A 120 minute treadmill run at 60% of vVO_{2max} interspersed with sprints at 90% of vVO_{2max} for the last 30 seconds of every 10 minutes, followed by a 5km time trial, a protocol previously shown to induce transient elevations in IL-6 (92), was utilised in order to quantify changes in the methylation of PBMCs, measured using the Infinium Human Methylation 27 microarray. 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.

Although research into the relationship between acute exercise, inflammation and epigenetic modification is clearly still in its infancy, and the plasticity of the observed effects remains to be established, the reviewed literature appears to support the notion that inflammation associated with acute exercise is likely to be a regulatory mechanism of changes in DNA methylation. This opens up an exciting new subdiscipline in exercise immunology, which may be mined for information beneficial not only to healthy and active individuals, but also to those suffering from a variety of disease states associated with chronic inflammation.

4.4 - Impact of Exercise Training and Physical Activity Interventions on Epigenetic Modification

Experimental manipulation of mode and intensity of exercise has begun to enhance our understanding of how the epigenome responds to prolonged periods of exercise training. For example, a six month training study (64) 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. However, methylation of the ASC gene, involved in IL-1β and IL-18 production (as described in section 3.1.3), 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 (14), 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 the well-documented age-related hypomethylation (2, 30, 37). Future studies focusing on gene-specific methylation may shed more light on this possibility.

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 (150min/week of moderate intensity aerobic exercise on a treadmill for experimental group; control group received only usual clinical care) altered the methylation profile of 43 genes (101). 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, RP11-450P7.3, 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 of particular interest in the context of cancer; L3MBTL1, a candidate tumour suppressor gene (34, 76), 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 (77) that considerable variation exists between PBMCs and granulocytes, and even within each cell population (T cells/natural killer/B cells/monocytes), a variation that is considerably more pronounced in adult blood than cord blood (45). 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 it is unlikely that blood will 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 (49, 75).

Exactly what type of exercise is optimal to achieve these beneficial effects in the context of cancer has been only partially elucidated. Bryan et al (10) 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 VO_{2max}, when compared with the control group. At baseline, average methylation of the selected CpG sites was inversely correlated with PAR (7 Day Physical Activity Recall) minutes, which remained significant after controlling for age, but not BMI. Following the intervention, the increase in PAR score was significantly correlated with a decrease in methylation, even after controlling for age, BMI and baseline VO_{2max}, highlighting that chronic PA may convey protective effects due to inhibition of DNMT activity that may result in aberrant DNA methylation at particular sites which could promote tumorigenesis.

Turning attention now to another globally relevant disease, T2DM patients could also potentially benefit from exercise-related epigenetic modulation. A controlled study on a cohort of individuals with a family history of T2DM indicates that a six-month exercise intervention was sufficient to induce alterations in both global and gene-specific methylation, independent of family history of T2DM (69). Overall, vastus lateralis skeletal muscle biopsy 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 (48, 61), THADA, associated with T2DM (60), and NDUFC2, which encodes NADH hydrogenase, the first enzyme of the oxidative phosphorylation system within the mitochondrial inner membrane (95), were reported following the intervention. Additionally, methylation of IL-7, 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 (80) demonstrated that the exercise intervention resulted in global adipose tissue hypermethylation, decreased abdominal adiposity and diastolic blood pressure, and increased VO_{2max} and HDL. In addition to the "crude" assessment of global methylation, more than likely performed to enable comparison of results with existing literature, more 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 (36), as well as KCNQ1 and TCF712, which have both been implicated in the pathogenesis of T2DM (60, 87). 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.

Not all studies show exercise training programmes to have an effect on DNA methylation (20), although this is more than likely due to a number of methodological issues that have previously been discussed within this paper. The majority of studies reviewed here indeed agree that a period of six to 12 months is sufficient to modify gene-specific methylation of a number of different genes associated with pathologies such as aging, 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.

5.0 – CONCLUSION AND FUTURE DIRECTIONS

Although the aforementioned studies have begun to characterise the epigenetic response associated with exercise and inflammation, much of the available research has been conducted in the context of pathologies with an inflammatory component. For the scientific community to achieve a thorough understanding of the relationship between exercise, inflammation and the epigenome, we propose that a collaborative interdisciplinary approach is utilised. Research into this relationship is made more complex by the apparent interchangeable roles of inflammation and DNA methylation as the causative factor; on one hand, hypermethylation can cause upregulation of inflammation-associated genes, while on the other hand, pro-inflammatory cytokines can regulate expression of DNA methyltransferases. It remains to be clarified whether the inflammatory state associated with intense exercise causes detrimental modifications to the epigenome; *in vitro* studies suggest that this could be the case, which could potentially predispose athletes to disease. Conversely, since regular moderate exercise is known to reduce chronic inflammation, the health beneficial effects of regular exercise may be due in part to favourable epigenetic changes. This, therefore, suggests that there is potential for novel epigenetic-based preventative and therapeutic strategies through non-pharmacologic methods such as lifestyle manipulation.

From our review of the literature, a few points to consider in the technical design of an epigenetic study have also become evident. Firstly, it is clear that the original method of global methylation assessment is not sufficient in isolation, and that gene-specific analyses are now required to provide accurate information that adds to the scientific knowledge base, particularly when studying epigenetic markers that increase susceptibility to a disease. Secondly, future research should attempt to identify regions in the genome which may be particularly susceptible to epigenetic modification in response to exercise, and to investigate to what extent differences in activity type, duration or intensity may yield differential effects. Thirdly, it has been shown that variation of DNA methylation is greater between tissues (liver, heart and kidney) than between species (human and chimpanzee) (75), while another study (49) reported that CpG methylation of a single gene varies between muscle, kidney, colon, heart and brain. Even within blood, considerable variation exists between cells (45, 77). These findings highlight the need to look at tissue- and even cell-specific differences. Fourthly, it is important to keep in mind that although epigenetic modification such as DNA methylation is most probably a dynamic phenomenon, both the longevity and degree of reversibility of these adaptations are largely unknown at this stage. In the context of disease, reversibility has been demonstrated by the treatment of cutaneous T cell lymphoma with HDAC inhibitors, and myelodysplastic syndrome with DNMT inhibitors, which comprise the first generation of epigenetic drugs to be approved (58). However, in the absence of pharmacological intervention, as dis-

Study	Population	Activity	Measurement	Results
Habitual PA				
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.
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.
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, systolic and diastolic blood pressure, LDL and HDL cholesterol, serum triglycerides, smoking status and BMI).
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.
Shaw et al (2014)	253 white males, 137 white females. Elderly.	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.
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.	Differential methylation of 6 CpG sites in the experienced, compared with beginner, group.
Disease-specific				
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.	Lifetime, previous 5 years, and previous year PA inversely correlated with PM of APC but not RASSF1A.
Yuasa et al (2009)	106 male/female primary gastric carcinoma patients.	Self-administered pre-cancer PA history questionnaire.	Methylation of 6 tumour-related genes; CDX2, BMP-2, p16, CACNA2D3, GATA-5, ER following tumour biopsy.	PA inversely correlated with CACNA2D methylation.
Acute Exercise				
Barrès et al (2012)	14 sedentary, healthy men/women.	Acute bout: cycle ergometer VO_{2peak} test.	GM of vastuslateralis 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.	Acute bout: 120 minutes of treadmill running at 60% vVO_{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.
Exercise Interventions				
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 VO_{2peak}, followed by 3 minutes of high intensity walking above 70% $VO_{2peak,}$ at least 2 days per week. Tracked by accelerometry.	Peripheral blood ASC gene methylation.	ASC methylation decreased with age, while the exercise intervention attenuated this age related decrease.
Zeng et al (2011)	12 women (6 = exercise intervention, 6 = control), BC diagnosis.	6 months of 150 minutes of moderate intensity treadmill exercise.	HumanMethylation27 Beadchip analysed 27,578 CpG sites in peripheral blood leukocyte/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.
Bryan et al (2013)	64 sedentary men/women, mean age = 29 years.	Psychologically tailored materials designed to increase PA participation over 12 months.	Methylation of 45 CpG sites from saliva samples.	Post-intervention, self-reported PA score inversely correlated with methylation (after controlling for age, BMI and baseline VO_{2max}).
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 vastuslateralis muscle biopsy.	Hypomethylation of 115 genes, and hypermethylation of 19 genes.
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.	Genome wide analysis (HumanMethylation450 Beadchip) of subcutaneous adipose tissue of the right thigh.	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.

PA = Physical Activity; BC = Breast Cancer; GM = Global Methylation; PM = Promoter Methylation; LINE-1 = Long Interspersed Nuclear Element 1; LUMA = Luminometric Methylation Assay; PCR = Polymerase Chain Reaction; PBMC = Peripheral Blood Mononuclear Cells; BMI = Body Mass Index; FH = Family History; T2DM = Type 2 Diabetes Mellitus; MeDIP = Methylated DNA Immunoprecipitation.

cussed elsewhere in this review in the context of glucocorticoid sensitivity, epigenetic changes appeared to persist relatively longer. The notion that exercise may potentially be able to reverse epigenetic-induced aberrations in gene expression associated with disease pathogenesis, thereby suppressing the disease state, is an exciting new avenue to pursue in exercise science. Finally, in terms of exercise, the available, published epigenetic studies have focused on therapeutic training interventions and anti-inflammatory outcomes, employing moderate intensity exercise training protocols. However, in contrast, the effect of strenuous or excessive exercise on epigenetic modulation has received scant attention leaving a potentially fruitful avenue for future researchers to investigate, and would allow more extensive characterisation of the precise relationship between exercise-induced inflammation and epigenetic regulation.

We have identified three important burning issues or questions that still need to be addressed within in this domain. Firstly, the optimal intensity, duration and mode of exercise that would elicit beneficial changes to the methylome needs to be established. Protocols used in training studies that have shown peripheral benefits in terms of inflammation are probably a good starting point. Secondly, to inform on potential target sites susceptible to epigenetic modification, the exact molecular mechanisms by which these changes are regulated needs to be elucidated. Thirdly, other factors contributing to the complexity of the exercise-inflammation relationship have not received much attention. For example, there is a close relationship between inflammation and oxidative stress, and thus, the possibility of this as another causative factor within the context of exercise, should be investigated. Similarly, the high-carbohydrate diet traditionally consumed by athletes is now associated with inflammation in the context of heart disease, but this potential role player has not been the focus of exercise studies with an epigenetic focus.

In conclusion, the need to further understand the effects of both unaccustomed and more moderate, habitual exercise on inflammation in the context of epigenetic mediators and signalling pathways is essential if we are to fully understand the way in which changes occur. With the application of considered, standardised techniques and study design, inclusion of an epigenetic approach to exercise-related research may add vital information that would otherwise have remained elusive.

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