EPIGENETICS
2019, VOL. 14, NO. 3, 294–309
https://doi.org/10.1080/15592294.2019.1582276

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RESEARCH PAPER

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

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ABSTRACT

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

ARTICLE HISTORY
Received 1 October 2018
Revised 25 January 2019
Accepted 7 February 2019

KEYWORDS
PPARGC1A; IL6; TNFa; DNMT; DNA methylation; exercise; inflammation; n-3 PUFA

Introduction

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

Acute exercise is associated with adjustments in the expression of genes involved in a variety of cellular processes, including immune response mitochondrial biogenesis, metabolism and muscle remodelling [14–16]. The *PPARGC1A* gene, which encodes for peroxisome proliferator-activated receptor gamma, co-activator alpha (PGC1-a), is known as the master regulator of mitochondrial biogenesis and plays an important role in aerobic training adaptation [17]. In immune cells, PPARGC1A is associated with anti-inflammatory [18,19] and anti-oxidant defence [20]; however, the impact of exercise-induced inflammation and oxidative stress on PPARGC1A DNA methylation is unknown. Epigenetic studies have linked a CpG site −260 bases from the promoter of *PPARCG1A* with the regulation of mRNA expression. In skeletal muscle, exercise can demethylate the

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PPARGC1A −260 CpG site which has been shown to concurrently upregulate *PPARGC1A* mRNA expression [8,10,21]. Although well characterised in skeletal muscle, the regulation of *PPARGC1A* expression in other cells and tissues, including immune cells is poorly understood [22].

Exercise of sufficient intensity and duration can cause tissue injury and lead to a systemic inflammatory response [14,23]. Increased circulating levels of the inflammatory cytokines IL-6 and TNFa are strongly correlated with the progression of sarcopenia and measures of physical performance [24,25]. Acute exercise can also increase the production of reactive oxygen species, in both skeletal muscle and immune cells [26], potentially leading to the development of oxidative stress and damage to lipids, proteins and DNA [27]. Increases in markers of oxidative stress and circulating levels of inflammatory cytokines, such as IL-6 and TNFa, have been shown to alter the expression of DNA methyltransferases (DNMTs) [28–32] and influence DNA methylation patterns [11,33]. DNA methylation of inflammatory cytokines have been associated with various inflammatory diseases including *IL6* with Rheumatoid Arthritis [34] and obesity [35]; *TNF* DNA methylation with type 2 diabetes [36] and Alzheimer's disease [37]. Despite increased circulating levels of inflammatory cytokines post-exercise [14,23], the impact of exercise on the DNA methylation of genes encoding inflammatory cytokines such as *IL6* and *TNF* remains unknown.

There is the potential for the dietary supplementation of fatty acids (FAs) to prevent the exercise-induced inflammation via the modulation of DNA methylation. Supplementation of FAs, including omega-3 polyunsaturated FAs (n-3 PUFAs) and extra virgin olive oil (EVOO), are consumed to reduce levels of inflammation [38,39], however, the impact of these supplements on exercise-induced inflammation is equivocal. Some studies have detected reductions in inflammation post-exercise with FA supplementation [40,41], whereas, others have reported no change in inflammation [42,43]. An emerging mechanism for the anti-inflammatory impact of FA supplementation is via epigenetic modifications [44–47]. The supplementation of the diet with krill oil, high in n-3 PUFAs, has been demonstrated to reduce PPARGC1A mRNA expression and the change in mRNA expression was negatively correlated to the change in plasma n-3 PUFAs [48]. Total n-3 PUFA content is negatively correlated to both IL6 DNA methylation and IL-6 protein concentration [47].

EVOO is a commonly used control in exercise studies to assess the impact of n-3 PUFA; however, the supplementation of EVOO has also been reported to modify the DNA methylation of genes associated with inflammation [49]. It remains to be identified whether the supplementation of FAs have an epigenetic impact on exercise-induced inflammation.

The present study investigated the impact of aerobic exercise on global and gene-specific (*PPARGC1A, IL6* and *TNF*) DNA methylation and *DNMT* mRNA expression in leukocytes of disease-free individuals. We also investigated whether these relationships could be modified by the supplementation of FAs. The association between physiological markers related to exercise performance, inflammation and oxidative stress post exercise and DNA methylation were also investigated.

Results

Global cytosine methylation and DNMT mRNA expression

One-hour of cycling reduced global methylation, assessed by the Luminometric Methylation Assay (LUMA; Figure 1(a); Pre 79.2%; Post 78.7%, $p = 0.008$), and the mRNA expression of both *DNMT3a* (Figure 1(c)); $p = 0.018$) and DNMT3b (Figure 1(d)); $p = 0.046$). Supplementation of FAs did not alter global methylation or mRNA expression of *DNMT3a* or *DNMT3b* (Figure 2; $p > 0.05$). While *DNMT1* mRNA expression was unaffected by exercise, a significant interaction was identified between supplement and trial ($p = 0.048$; Figure 2(b)) indicating differential effects on mRNA expression with the two supplements. No correlation was detected between global DNA methylation values and *DNMT* mRNA expression.

Gene-specific DNA methylation and mRNA expression

PPARGC1A

A reduction in *PPARGC1A* DNA methylation (Pre 6.9%; Post 6.3%, Figure 3(a); $p < 0.001$) and an increase in mRNA expression (Figure 3(b));

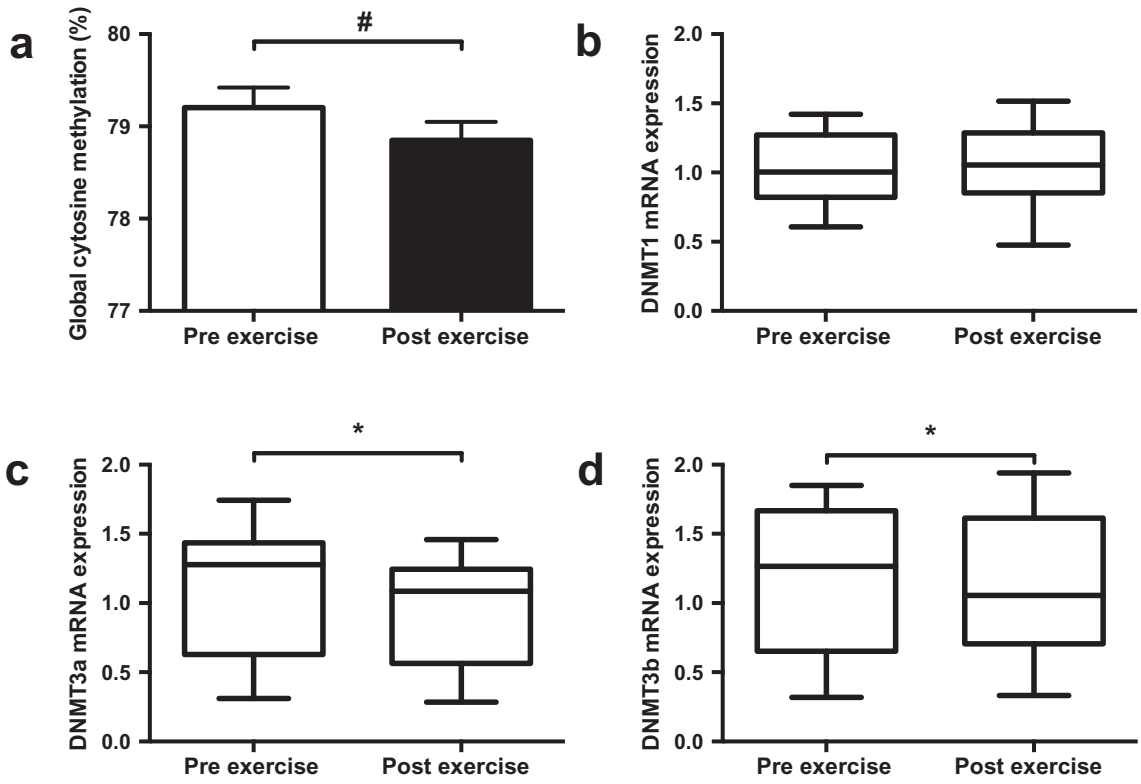

Figure 1. Effect of exercise on global DNA methylation (a) and mRNA expression of DNMT1 (b), DNMT3a (c) and DNMT3b (d). Data presented as the mean value of all trials for each time point. * $p < 0.05$, # $p < 0.01$.

$p < 0.001$) were detected following exercise. The supplementation of FAs had no impact on *PPARGC1A* DNA methylation or mRNA expression ($p > 0.05$). Moderate but non-significant negative correlations were detected between *PPARGC1A* DNA methylation and *DNMT3a* and *DNMT3b* mRNA expression (Figure 5).

IL6

Despite an increase in IL-6 protein concentrations following exercise (Pre: 0.63 ± 0.24 pg/mL, Post: 3.78 ± 0.55 pg/mL; $p < 0.001$), there was no change in *IL6* DNA methylation ($p > 0.05$) or mRNA expression ($p > 0.05$) following exercise. A significant interaction was detected between supplement and trial for CpG3 (−1094) indicating increased DNA methylation following n-3 PUFA and decreased methylation following EVOO (Figure 4(a)); $p = 0.038$). A similar, non-significant ($p = 0.080$) trend was detected for IL6 mRNA expression following supplementation (Figure 4(b)). A significant correlation was detected between the mean *IL6* methylation across all CpG sites and *DNMT3b* mRNA expression (Figure 5, $p = 0.007$).

TNF

Neither exercise or the supplementation of FAs altered *TNF* DNA methylation or mRNA expression. Trends were identified between 3 *TNF* CpG sites and differential methylation following supplementation (CpG2 $p = 0.069$; CpG3 $p = 0.098$; CpG4 $p = 0.067$; CpGmean $p = 0.077$). *TNF* DNA methylation was negatively correlated with *TNF* mRNA expression (Figure 5; $p = 0.007$). Moderate, however, non-significant correlations were detected between both *IL6* and *DNMT3a* mRNA expression, and TNF DNA methylation (Figure 5).

Associations between DNA methylation and post-exercise physiological markers

Figure 6 demonstrates the association between post-exercise DNA methylation and physiological markers related to exercise, oxidative stress and inflammation. Prior to FA supplementation, both *PPARGC1A* and *TNF* methylation post-exercise are significantly correlated with Time Trial (TT) performance (Figure 6, $p < 0.05$). Following the supplementation of n-3 PUFA and EVOO,

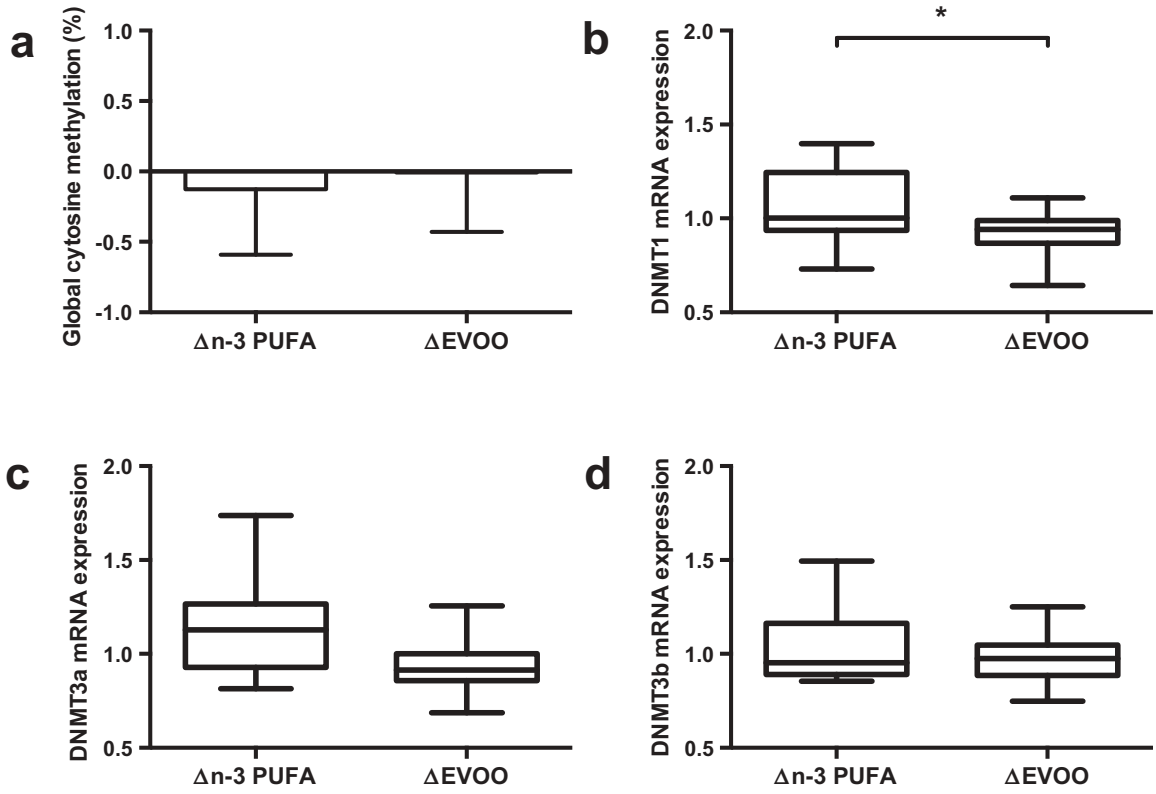

Figure 2. The impact of supplementation of n-3 PUFA and EVOO on global DNA methylation (a) and mRNA expression of DNMT1 (b), DNMT3a (c) and DNMT3b (d). Data presented as the relative change (Δ) between pre and post supplementation trials (post supplementation – pre supplementation) for each supplement. n-3 PUFA, n-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil. * $p < 0.05$.

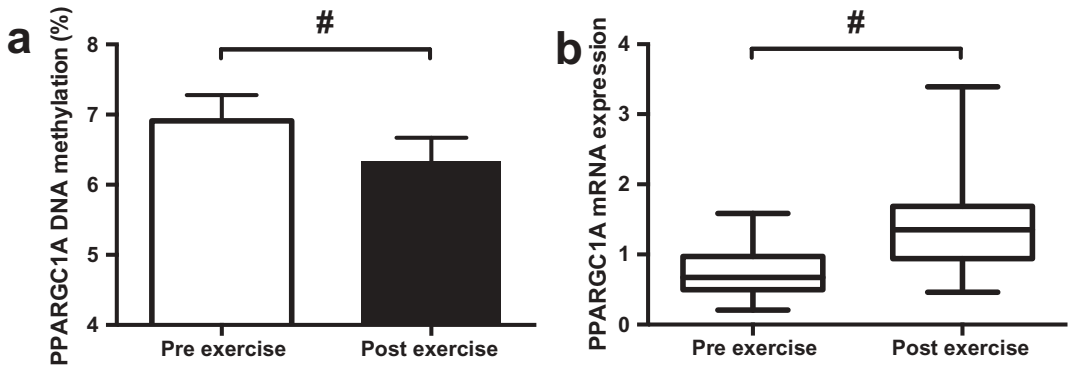

Figure 3. Effect of exercise on DNA methylation of CpG-260 (a) and mRNA expression (b) of PPARGC1A. Data presented as the mean value of all trials for each time point. # $p < 0.01$.

correlations between TT performance and both *PPARGC1A* and *TNF* DNA methylation are weakened and no longer significant (Figure 6). A negative correlation was detected between peripheral blood mononuclear cell (PBMC) protein carbonyl (PC) concentration, an intracellular measure of oxidative stress, and both global and PPARGC1A methylation prior to supplementation of FAs, however, no association was detected following n-3 PUFA supplementation (Figure 6). The concentration of PC in serum, a systemic measure of oxidative stress, was uncorrelated with DNA methylation at baseline, however, following EVOO supplementation significant correlations existed between serum PCs and both PPARGC1A and *TNF* DNA methylation (Figure 6). The only significant correlation between DNA methylation and serum IL-6 concentration was a negative correlation with global DNA methylation following n-3 PUFA supplementation (Figure 6).

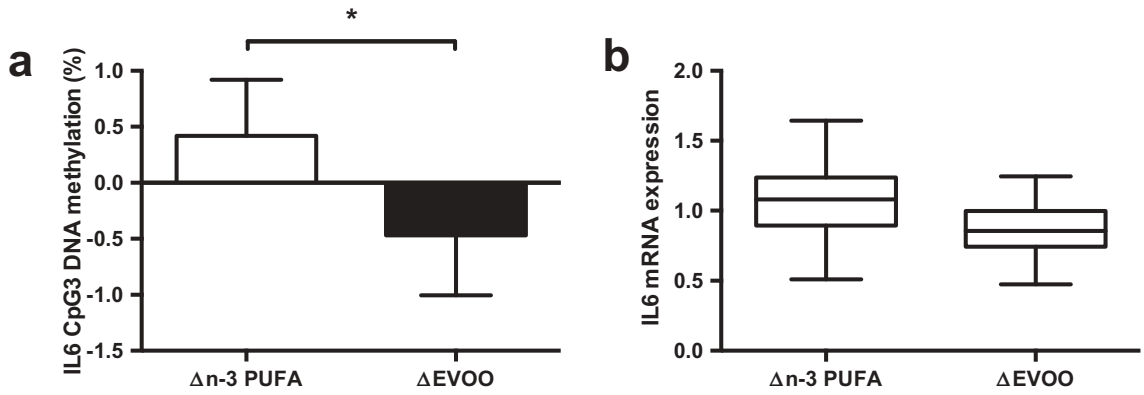

Figure 4. The impact of n-3 PUFA and EVOO supplementation on IL6 CpG3 DNA methylation (a) and IL6 mRNA expression (b). Data presented as the change (Δ) between pre and post supplementation trials (post supplementation – pre supplementation). n-3 PUFA, n-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil. * $p < 0.05$.

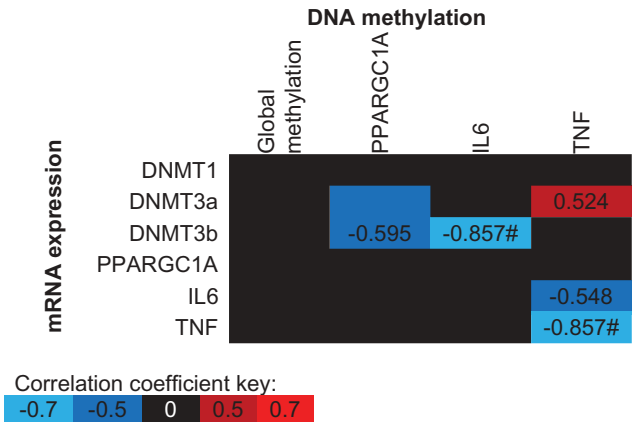

Figure 5. Spearman's Rho correlation coefficients between mean DNA methylation values and gene expression values across all conditions (supplement, time and trial). The mean of all CpG sites assessed for each gene has been used to provide an overall view of the region of interest. Blue indicates a negative correlation, red indicates a positive correlation and black indicates correlation coefficients between −0.5 and 0.5. * $p < 0.05$, # $p < 0.01$.

Discussion

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

For the first time we report, global hypomethylation in leukocytes following an acute bout of exercise. The only previous study to investigate the impact of acute exercise in blood cells failed to detect any change in DNA methylation following correction for multiple testing [11]. The results of the present study are in accordance with previous reports of a net hypomethylation following chronic exercise training [2–7] and acute bouts of exercise in plasma [50] and skeletal muscle [4,8]. Other studies have failed to detect any change in global DNA methylation [51,52]; however, this can be explained by a similar number of CpG sites increasing and decreasing in DNA methylation [51]. It has also been demonstrated that exercise-induced hypomethylation is retained during periods of detraining, allowing it to become further hypomethylated following further training [4]. These data suggest that both acute and chronic exercise is sufficient to alter DNA methylation patterns typically resulting in hypomethylation.

In the present study, a 4-week supplementation of FAs did not influence global DNA methylation. In contrast, a 6-month supplementation of n-3 PUFA decreased *LINE-1* DNA methylation, a surrogate for global DNA methylation, in Alzheimer's patients [53].

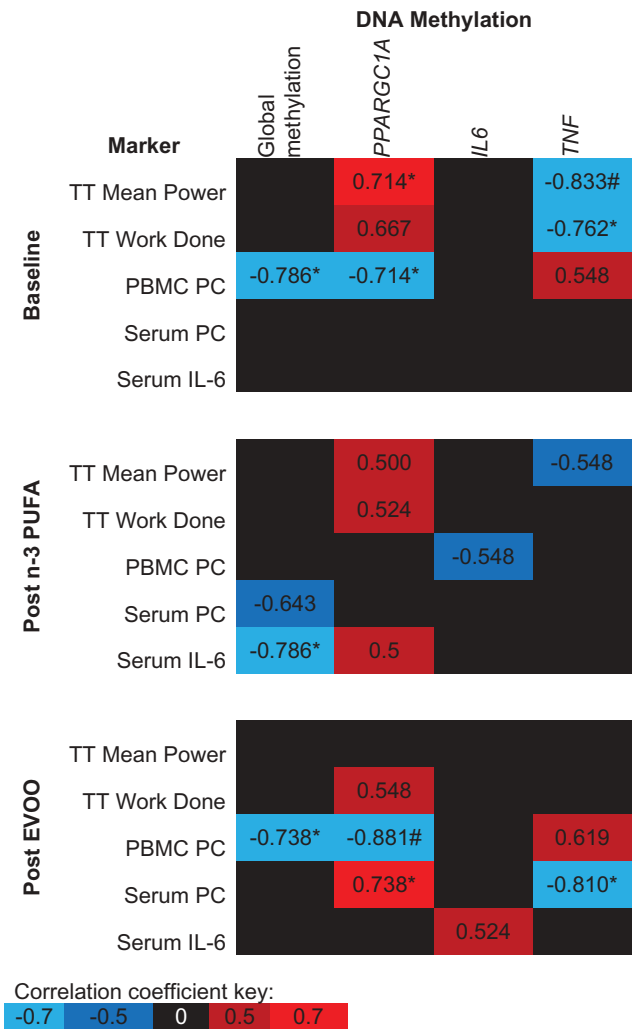

Figure 6. Spearman's Rho between post-exercise DNA methylation and physiological markers related to exercise performance, oxidative stress and inflammation. The mean of all CpG sites assessed for each gene has been used to provide an overall view of the region of interest. Blue indicates a negative correlation, red indicates a positive correlation and black indicates correlation coefficients between −0.5 and 0.5. n-3 PUFA, omega-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil; TT, Time trial; PC, protein carbonyl. *p < 0.05, # p < 0.01.

However, *LINE-1* methylation is increased in Alzheimer's patients compared to healthy controls [54], therefore, the supplementation of n-3 PUFA in these individuals may act to restore global DNA methylation to the normal level detected in healthy individuals. The use of different surrogate measures of global methylation (LUMA vs *LINE-1*) prevents the direct comparison between studies because of the different region which these assays investigate. Two separate studies have indicated that the methylation estimates provided by LINE-1 and LUMA are poorly correlated [55,56].

For the first time, decreased methylation and concurrent increased mRNA expression of *PPARGC1A* following a bout of aerobic exercise have been detected in leukocytes. The results from the present study match previous reports of aerobic exercise-induced hypomethylation in skeletal muscle [2,8,10] potentially indicating a systemic impact of exercise on *PPARGC1A* DNA methylation. The mRNA expression profile of skeletal muscle and PBMCs have been shown to be highly associated following an 8-week supplementation of n-3 PUFAs [57]. Although we do not find any association with *PPARGC1A* methylation/mRNA expression and n-3 PUFA supplementation in the present study, the hypomethylation detected in the present study is consistent with the impact of exercise in skeletal muscle providing further evidence for blood-derived expression profiles to be used as a surrogate for skeletal muscle.

The only previous report of *PPARGC1A* methylation from leukocytes failed to detect an association with physical activity [58]. The lack of previous association could be the result of the investigation of different CpG sites in the promoter region of *PPARGC1A*. Alternatively, the discordance in these results could reflect the heterogeneity in methylation pattern of immune cells [59]. Exercise increases the number of circulating leukocytes, therefore, changes in methylation may be the result of different proportions of leukocytes rather than a change in DNA methylation patterns [60]. The present study has adjusted DNA methylation values to account for the number of leukocytes (lymphocytes, neutrophils, monocytes, basophils and eosinophils) [59], whereas, previous reports have failed to account for this critical variable.

The positive correlation between leukocyte *PPARGC1A* methylation and exercise performance indicates that increased DNA methylation may provide a performance advantage. *PPARGC1A* is thought to upregulate mitochondrial biogenesis in monocytes to induce a shift towards an anti-inflammatory phenotype [18,19] and antioxidant defence in lymphocytes [20]. Although we did not find an association with IL-6 protein concentration, a negative association was detected between

PPARGC1A DNA methylation and PC concentration indicating epigenetic control of the antioxidant role of *PPARGC1A*. There is limited literature comparing mitochondrial function in leukocytes and skeletal muscle following exercise; however, the association between gait speed and mitochondrial function in both skeletal muscle tissue and PBMCs provides a conserved mechanism between mitochondrial function in skeletal muscle and blood-derived mitochondria [61]. Further evidence of a conserved mechanism is suggested with genes related to mitochondrial structure and function found to be co-expressed in skeletal muscle and neutrophils following aerobic exercise [62]. Future studies are required to detect if the same phenotypic associations exist in skeletal muscle as detected in leukocytes in the present study.

Aerobic exercise did not alter the DNA methylation or mRNA expression of either *IL6* or *TNF*. The epigenetic impact of exercise on inflammatory cytokines is relatively unknown, however, several studies have indicated a role for cytokine DNA methylation in inflammatory disease [34–37]. Although no association between *TNF* DNA methylation and mRNA expression was detected in the present study, n-3 PUFAs have previously been demonstrated to reverse the epigenetic changes observed with inflammation in skeletal muscle cells. The administration of TNF induced hypermethylation and decreased mRNA expression of MyoD [63], whereas the supplementation of EPA dampens the impact of TNF in muscle and restores MyoD mRNA expression [44]. Despite an increase in the circulating protein concentration of IL-6 in the present study, the exercise bout may have not increased TNFa protein concentration and induced an inflammatory response sufficient to modify DNA methylation patterns of inflammatory cytokines. *TNF* hypermethylation is reported in elderly individuals who maintained or increased their energy expenditure by 500 kcal/wk over an 8-year period compared to those who decreased energy expenditure over the same period [64]. The same *TNF* CpG sites as the present study have previously been shown to negatively associate with mRNA expression, plasma concentrations and measures of adiposity [65,66]. In the present study, a significant negative correlation was detected between *TNF* DNA methylation post-exercise and BMI, exercise performance and *TNF* mRNA expression. These data suggest an acute bout of exercise may not regulate *TNF* DNA methylation, however, the long-term benefits of regular exercise, such as reduced adiposity, may subsequently increase *TNF* DNA methylation levels and as a result, reduce *TNF* mRNA expression and the chronic low-grade inflammation levels associated with increased adiposity.

Previously decreased methylation in a region ~600 bp upstream of the *IL6* promoter has been associated with increased erythrocyte n-3 PUFA concentrations and mRNA expression [47]. In the present study, the supplementation of EVOO and n-3 PUFA had contrasting effects on a single CpG (−1094) of *IL6* (increased methylation following n-3 PUFA and decreased methylation with EVOO). The region ~1,000 bp from upstream of was investigated in the present study because of previous associations between DNA methylation and both inflammatory diseases [34,35] and mRNA expression [34]. Conflicting results between studies may indicate that distinct regions of the promoter regulate *IL6* expression differently. Supplementation of n-3 PUFA and OO have been shown to induce differential methylation of elongase and desaturase enzymes which are responsible for the metabolism of FAs [67]. The differential DNA methylation of these enzymes indicates the potential for n-3 PUFAs to switch towards the production of less inflammatory eicosanoids. Although the DNA methylation of desaturase and elongase enzymes have not been measured in the present study, a switch towards n-3 PUFA derived eicosanoid production, such as 3-series rather than 2-series prostaglandins, has been shown to reduce cytokine expression [38] which is potentially indicated by the increased DNA methylation of *IL6* following n-3 PUFA, but not EVOO, supplementation.

The impact of exercise and FA supplementation on *DNMT* mRNA expression was investigated to identify whether changes in *DNMT* mRNA expression could be a potential mechanism underlying modulated DNA methylation. *DNMT1* mRNA expression was modulated by FA supplementation, whereas, exercise reduced the expression of both *DNMT3a* and *DNMT3b*. This is the first demonstration of reduced expression of *DNMT3a* following acute exercise, whereas, the reduction in *DNMT3b* expression has previously been reported [31,68]. The inclusion of DNA methylation assessment in the present study allows the confirmation

that following a single bout of aerobic exercise *DNMT* expression is decreased alongside decreases in global and gene-specific DNA methylation. The only previous report of concurrent assessment of exercise-induced *DNMT* expression and DNA methylation was following an 8-week resistance training program [6]. The genome-wide method of methylation does not identify a net increase or decrease in global methylation; therefore, further studies are required to identify whether the modulation of *DNMT3b* causes hypomethylation or if it is important in both hyper- and hypomethylation.

The present study detects contrasting effects of n-3 PUFA and EVOO supplementation on *DNMT1* mRNA expression. There is a paucity of literature surrounding the impact of FA supplementation on *DNMT* expression in humans, whereas, animal models have associated supplementation of alpha-linolenic acid supplementation, a n-3 PUFA, with changes in *DNMT* mRNA expression [69,70]. Interestingly, similar to the present study, no change in global DNA methylation was detected alongside modulated *DNMT1* expression [69]. A change in global DNA methylation potentially would not be expected with increased in *DNMT1* mRNA expression because *DNMT1* functions to maintain DNA methylation. The impact of EVOO on *DNMT* expression is unknown, however, EVOO contains phenolic compounds, including decarboxymethyl oleuropein aglycone (DOA) [71], which reduce DNMT activity via competitive inhibition [72]. The absence of a measure of DNMT activity is a limitation of the present study, however, parallel changes in DNMT mRNA expression and activity have previously been reported [73]. A measure of activity could potentially explain the lack of association between altered *DNMT* mRNA expression and modulated DNA methylation following supplementation which should be considered in future studies.

While exercise and FA supplementation may directly influence *DNMT* expression, these interventions may modulate *DNMT* expression by intermediary mechanisms. The expression of several miRNAs, including miRNA-29 −130 and −148, are associated with: DNMT expression [74–77], exercise [78] and FA supplementation [79–81]. IL-6 protein levels have been reported to regulate *DNMT* mRNA expression [30–32] via the modulation of miRNA [29]. The small increase in IL-6 protein expression following exercise in the present study may be insufficient to modulate *DNMT* expression explaining the lack of agreement with previous reports. Future studies should use a bout of exercise with a greater inflammatory response, such as eccentric exercise, to examine the effect of exercise-induced inflammation on *DNMT* expression. The capability of exercise and n-3 PUFA supplementation to modify the expression of the same miRNAs which control the expression of *DNMTs* suggests miRNA expression could be one of the underlying mechanisms controlling DNA methylation.

The use of a homogenous population of trained cyclists in the present study potentially limits the generalisability of the results to other populations. Trained male cyclists were selected as the population for the present study because they are the most familiar with the exercise stimuli and we would expect this to reflect in the smallest epigenetic response. Previously a single bout of exercise was sufficient to reduce global DNA methylation in plasma of COPD patients; however, following a training intervention the exercise bout was no longer sufficient to reduce global DNA methylation [50]. Exercise training has previously been demonstrated to alter DNA methylation patterns differently depending on family history of diabetes [2]. Future studies should compare the impact of exercise in trained athletes and sedentary individuals or a disease cohort to determine whether exercise-induced alterations to the DNA methylome are contributors to health and disease in diverse populations.

In conclusion, the present study highlights the impact of an acute bout of aerobic exercise and the supplementation of FAs on DNA methylation and mRNA expression in leukocytes of trained male cyclists. Alterations in the epigenetic control of these genes are associated with physiological markers related to exercise performance and inflammation/oxidative stress, however, a more extensive study is required to confirm these associations. The observational nature of the present study prevents the identification of the underlying mechanisms controlling altered DNA methylation following exercise and FA supplementation, therefore, future mechanistic studies are required to identify such mechanisms. Here

we suggest that modulation of DNMT mRNA expression may be one such mechanism for future research. Future studies should compare multiple tissue types to examine whether exercise and supplementation of FAs have systemic effects on DNA methylation.

Methods

Participants

Complete sets of data were available for eight participants whose characteristics are described in Table 1. Prior to participation, informed written consent was provided by each participant. Participants were healthy, non-smokers with no history of metabolic or cardiovascular disease. In the six-months prior to the study, participants had no history of n-3 PUFA, anti-oxidant or anti-inflammatory supplementation. Participants recorded their physical activity and maintained habitual diet throughout the study. The experimental protocol was approved by the Loughborough University Ethics Human Participants sub-committee and performed in accordance with the Declaration of Helsinki 1975.

Study overview

The study consisted of a pre-test and four experimental trials. Experimental trials were completed before and after a four-week supplementation of n-3 PUFA and EVOO in a double-blind, randomised, repeated measures design. A four-week washout was included between each supplementation period (Figure 7).

Pre-test

Participants underwent anthropometric assessment for height, body mass and eight-skinfold measurements prior to the start of the study. Maximal aerobic work rate (Wmax) and maximal oxygen uptake ($\dot{V}O_{2max}$) were determined using a graded exercise test on a Lode Excalibur Sport ergometer (Lode B.V, Netherlands). The exercise test began with a warm-up period of 5-min cycling at 100 W. Workload then increased by 50 W every 3-min until volitional fatigue (decrease in self-selected cadence of 20 revs·min^{-1}). Expired air was collected in the final minute of each stage to allow $\dot{V}O_{2max}$ determination using primary and secondary criteria [82]. Wmax was calculated using the formula:

$$\text{Wmax} = \text{Workload} \div [(t/180)\text{x}50]$$

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

Table 1. Participant characteristics. Wmax, maximal aerobic work rate.

Variable	All participants (n = 8)
Age (yrs)	39.50 ± 5.90
Body Mass (kg)	73.04 ± 8.31
Height (cm)	174.26 ± 8.41
Wmax (W)	321.63 ± 28.15
$\dot{V}O_{2max}$ (mL·kg·min^{-1})	53.88 ± 5.24

Experimental trials

Trials were conducted in the morning (7–9 am) following a 10-hour overnight fast. Participants were asked to complete a 3-day food diary, refrain from strenuous exercise and the consumption of alcohol or caffeine for the 24-hours prior to the trial. The performance test consisted of 45-minutes cycling at 70% Wmax, followed by a 15-minute TT [83].

Supplementation

Both n-3 PUFA (Holland and Barrett, Warwickshire, UK) and EVOO (Puritan's Pride, New York, USA) supplements were provided in capsule form. Participants were instructed to take 6 capsules per day providing 5.7g of n-3 PUFA and 0.01g per day of α-Tocopherol or 6 g per day of EVOO. The n-3 PUFA dose was chosen based on previous findings showing the dose was sufficient to induce changes in the lipid profile of human blood over four weeks [84,85]. Compliance of supplementation was monitored by capsule counts.

Analytic procedures

Blood sampling

Venous blood was sampled via an intravenous catheter inserted into an antecubital vein of the non-dominant arm for the collection of whole blood pre and immediately post-exercise (Figure 7) for DNA methylation analysis, mRNA expression and a whole blood cell count using the COULTER® Ac·T™ 5diff (Beckman Coulter, UK). PBMCs were isolated from

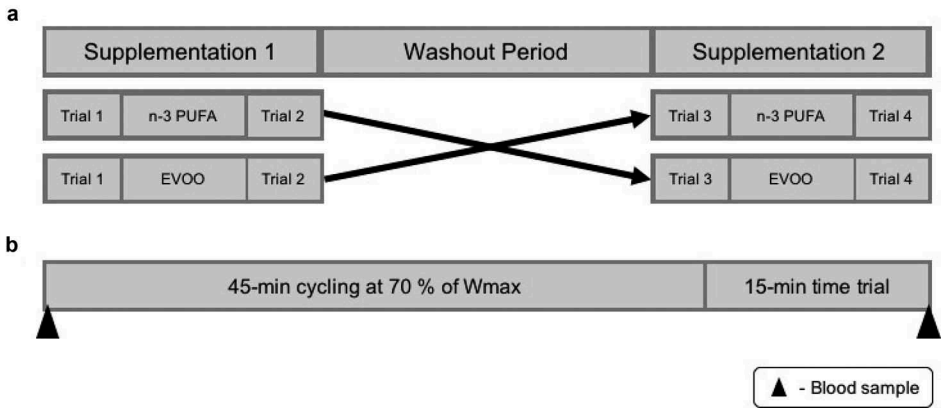

Figure 7. Schematic representation of study outline (a) and trial day (b). n-3 PUFA, omega-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil; Wmax, maximal aerobic work rate.

whole blood by density gradient centrifugation using Ficoll-Paque Premium (GE healthcare, USA) according to manufacturer's instructions. The resulting PBMC cell pellet was suspended in 200µl RIPA buffer for analysis of protein carbonyls. Whole blood collected in vacutainers (Becton, Dickson & Company, UK) that contained no anticoagulant was allowed to clot at room temperature and centrifuged at 2800 rpm for 15 minutes for analysis of serum protein carbonyls and IL-6.

Nucleic acid isolation

Genomic DNA (gDNA) was isolated from 2mL of whole blood using the QIAamp DNA Blood Midi kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was isolated from whole blood collected in Tempus Blood RNA tubes using the Tempus Spin RNA Isolation Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The concentration (mean ± SD) and purity (absorbance ratio A_{260}/A_{280} ± SD) of isolated DNA and RNA were determined using a Nanodrop 2000 (ThermoScientific, USA). The mean concentration of isolated gDNA was 183.50 ± 54.48 ng/µL with a A_{260}/A_{280} ratio of 1.90 ± 0.02, whereas, RNA concentration was 120.32 ± 41.02 ng/µL with an A_{260}/A_{280} ratio of 2.09 ± 0.02. Following extraction, DNA and RNA were stored at −20°C and −80°C respectively.

Luminometric methylation assay

LUMA was used as a marker of global DNA methylation as previously described [86], with minor adjustments. Briefly, two reactions containing 200 ng of gDNA were set up per sample, one with the methylation-sensitive enzyme FastDigest HpaII and one FastDigest MspI (Thermo Scientific, USA) and incubated for 20 min at 37 °C. Following incubation, 13 µL of each reaction were mixed with annealing buffer and added to a separate well of a Pyromark Q24 plate and analyzed using a PyroMark Q24 MDx system (Qiagen, Germany) with the following dispensation order: ACTCGA. Peak heights were exported, and methylation percentage was calculated using the following formula:

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

Bisulfite pyrosequencing

gDNA samples were bisulfite converted using the EpiTect Fast Bisulfite Conversion Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR of bisulfite converted DNA samples was performed using the PyroMark PCR Kit (Qiagen, Germany) according to the manufacturer's instructions. For all assays, an initial activation period of 15 min at 95°C was followed by a 3-stage cycling process of denaturation (95°C for 30s), annealing (56°C for 30 s) and extension (72°C for 30 s) for 45 cycles. The PCR process was finished with a final extension period of 72°C for 10 min. Pyromark custom assay (Qiagen, Germany) genomic location, primer sequences and the sequence to analyze are presented in Table 2. To confirm a single PCR product, amplicons were analyzed by gel electrophoresis and visualised by ultraviolet trans-illuminator (BioRad, USA). The absence of PCR amplification of non-bisulfite converted DNA confirmed the specificity of each assay for bisulfite converted DNA. DNA

Table 2. Details of pyrosequencing assays used to determine DNA methylation. Genomic location identified using genome reference consortium human build 38 patch release 12. CpG sites are indicated in the sequence to analyze by **Y**. For, forward primer; Rev, reverse primer, Seq, sequencing primer; TSS, transcription start site; bp, base pair.

Assay ID [Genomic location]	Primer	Sequence	No. of CpG sites (distance from TSS; bp)
PPARGC1A	For:	5′-TGTAGGGGATTTTGGTTATTATATGGT-3′	1
[chr4:23,890,308–23,890,372]	Rev:	5′-biotin-ACCAACTTTAAATACCACAAACTCTA-3′	(−260)
	Seq:	5′-GGTTATTATATGGTTAGGGT-3′	
	Sequence to analyze:	TT**Y**GTTTAGAGTTTGTGGTATTTAAAGTT	
IL6	For:	5′-GGGAAGAGGGTTTTTGAATTAG-3′	6
[chr7:22,726,051–22,726,198]	Rev:	5′-biotin-CTCCCTCTCCCTATAAATCTTAATTTAA-3′	(−1099, −1096, −1094,
	Seq:	5′-TTGAATTAGTTTGATTTAATAAGAA-3′	-1069, −1061 & −1057)
	Sequence to analyze:	ATTTTTGGGTGT**Y**GA**Y**G**Y**GGAAGTAGATTTAGAGTTTAGAG T**Y**GTGTTTG**Y**GTT**Y**GTAGTTTTTTTTTAGTTTTTTTTGATTT	
TNF	For:	5′-GGAAAGGATATTATGAGTATTGAAAGTATG-3′	4
[chr6:31,575,730–31,575,816]	Rev:	5′-biotin-CTAAAACCCCCCTATCTTCTTAAA-3′	(+197, +202, +214 &
	Seq:	5′-ATTATGAGTATTGAAAGTATGAT-3′	+222)
	Sequence to analyze:	T**Y**GGGA**Y**GTGGAGTTGGT**Y**GAGGAGG**Y**GTTTTTTAAGAA GATAGGGGGGTTT	

methylation was assessed using a PyroMark Q48 Autoprep system (Qiagen, Germany) using Pyro Mark Q48 Advanced CpG Reagents (Qiagen, Germany). The nucleotide dispensation order was generated by entering the sequence to analyze into the PyroMark Q48 Autoprep software version 2.4.2 (Qiagen, Germany). A non-CpG cytosine was included in the nucleotide dispensation order to detect incomplete bisulfite conversion. The methylation at each CpG site was determined using the PyroMark Q48 Autoprep software set in CpG mode. The mean methylation of all CpG sites within the target region was determined using the methylation at the individual CpG sites. Standards of known methylation percentages (0%, 12.5%, 25%, 50%, 75%, 87.5%, 100%) were created using the EpiTect PCR control DNA set (Qiagen, Germany) and underwent pyrosequencing analysis to generate standard curves between the expected and observed methylation percentage to check the assays for PCR bias. A high coefficient of determination ($R^2 > 0.99$) was determined for each assay indicating the absence of PCR bias.

mRNA expression

A minimum of 1 μg of RNA was reverse transcribed into complementary DNA (cDNA) using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, USA) according to the manufacturer's instructions and diluted to a concentration of 5 ng/μL in deionised water. Relative mRNA expression was performed by quantitative PCR (qPCR) for each gene of interest and normalised to the expression of *GAPDH* using a Viia7 Real-Time PCR system (Applied Biosystems, USA). Each reaction contained 5 μL of SybrGreen PrecisionPlus qPCR Master Mix (PrimerDesign, UK), 0.5 μL of forward and reverse primer (Table 3) and 4 μL of 5 ng/μL cDNA. All samples were run in duplicate using the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Melt curves were visually inspected for a single peak indicating the generation of a single product. The relative mRNA expression of the genes of interest were calculated using the $2^{-(\Delta\Delta Ct)}$ formula; the pooled group mean pre-exercise Ct from the initial trial was used as the control. The mean Ct value of *GAPDH* across all participants and experimental conditions was 17.13 ± 0.41 with low variation of 2.40%. The efficiency of each mRNA expression assay was determined (Table 3) using standard curves generated from a serial dilution of a cDNA sample. The efficiency was calculated using the formula:

$E = \left(\left(10^{(-1/\text{slope})}\right) - 1\right) \text{x} 100$, where the slope is the gradient of the linear regression fitted to the standard curve. The efficiency of each assay was between 90 and 105% with a $R^2 > 0.99$.

Interleukin-6 (IL-6)

Serum IL-6 concentrations prior to and immediately post-exercise were determined using high sensitivity enzyme immunoassay kits (R & D Systems, USA). Haematocrit and haemoglobin were used to ascertain plasma volume changes that were used to adjust serum IL-6 values [87].

Table 3. ` Details of assays used to determine mRNA expression. For, forward primer; Rev, reverse primer; bp, base pairs.

Assay ID	Accession No.		Sequence	Product length (bp)	PCR efficiency (%)
GAPDH	NM_001289745.2	For:	5′- GCCTCAAGATCATCAGCAATGCCT-3′	104	98.1
		Rev:	5′- TGTGGTCATGAGTCCTTCCACGAT-3′		
PPARGC1A	NM_001330751.1	For:	5′-CAGCCTCTTTGCCCAGATCTT-3′	101	104.0
		Rev:	5′-TCACTGCACCACTTGAGTCCAC-3′		
IL6	NM_000600.4	For:	5′-GCAGAAAAAGGCAAAGAATC-3′	178	100.9
		Rev:	5′-CTACATTTGCCGAAGAGC-3′		
TNF	NM_000594.3	For:	5′-AGGCAGTCAGATCATCTTC-3′	142	99.5
		Rev:	5′- TTATCTCTCAGCTCCACG-3′		
DNMT1	NM_001130823.2	For:	5′-TACCTGGACGACCCTGACCTC-3′	103	94.5
		Rev:	5′-CGTTGGCATCAAAGATGGACA-3′		
DNMT3a	NM_175629.2	For:	5′-TATTGATGAGCGCACAAGAGAGC-3′	111	95.9
		Rev:	5′-GGGTGTTCCAGGGTAACATTGAG-3′		
DNMT3b	NM_006892.3	For:	5′-GGCAAGTTCTCCGAGGTCTCTG-3′	113	96.2
		Rev:	5′-TGGTACATGGCTTTTCGATAGGA-3′		

Protein carbonyls (PC)

PC was assessed by an in-house ELISA [88,89]. Serum samples, PBMC lysates and standards were diluted in coating buffer (50mM sodium carbonate, pH = 9.2) to a concentration of 0.05mg/mL using the bicinchoninic assay method. Protein carbonyls groups were derivatised with 2, 4-dinitrophenylhydrazine (1mM, in 2M HCl) and incubated with monoclonal mouse anti-DNP antibody (Sigma Aldrich, UK) and rat anti-mouse IgE, conjugated to HRP (AbD Serotec, UK). Well absorbance was measured at 490nm and the PC concentration determined by using absorbance values of known PC standards made in our laboratory (1.28–5.20 nmol/mg protein). PC concentration in PBMCs was adjusted for changes in protein concentration and cell number (Beckman Coulter, UK) induced by acute exercise.

Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics software (SPSS version 23). The data were assessed for normality by Shapiro-Wilk's test. The composition of white blood cells from which the DNA is extracted is an important consideration in DNA methylation research; therefore, all DNA methylation analysis was conducted on cell heterogeneity adjusted values [59]. Analysis of mRNA expression was performed on log fold change data. DNA methylation and mRNA expression values were analyzed using a 2 (supplement) x 2 (trial) x 2 (time) repeated measures ANOVA. The impact of exercise is presented using the absolute values (mean of all trials for each time point), whereas, the impact of supplementation of FAs is presented as the relative change (Δ) between pre and post supplementation trials (post supplementation – pre supplementation). Values represented as mean ± 95% CI.

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

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by Loughborough University, School of Sport Exercise and Health Sciences and the NIHR Leicester Biomedical Research Centre.

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