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

David John Hunter [1,2], Lynsey James [1,2], Bethan Hussey [1,2], Alex J. Wadley [2,3], Martin R. Lindley [1,2] & Sarabjit S. Mastana [1,2]

Author Affiliations:

[1]Translational Chemical Biology Research Group, School of Sport, Exercise and Health Sciences, Loughborough University, Epinal Way, Loughborough, LE11 3TU, UK

[2] National Centre for Sport and Exercise Medicine, School of Sport, Exercise and Health Sciences, Loughborough University, Epinal Way, Loughborough LE11 3TU

[3] University Hospitals of Leicester NHS Trust, Infirmary Square, Leicester LE1 5WW

Corresponding Author:
Dr Martin R Lindley
School of Sport, Exercise & Health Sciences
Clyde Williams Building
Loughborough University
Loughborough, UK, LE11 3TU
Email: M.R.Lindley@lboro.ac.uk
Phone: 01509 226349

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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.

Keywords: PPARGC1A, IL6, TNFa, DNMT, DNA methylation, exercise, inflammation, fatty acid, n-3 PUFA.

58 **Introduction**

59

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

70

71 Acute exercise is associated with adjustments in the expression of genes involved in
72 a variety of cellular processes, including immune response mitochondrial biogenesis,
73 metabolism and muscle remodelling [14–16]. The *PPARGC1A* gene, which encodes
74 for peroxisome proliferator-activated receptor gamma, co-activator alpha (PGC1-a),
75 is known as the master regulator of mitochondrial biogenesis and plays an important
76 role in aerobic training adaptation [17]. In immune cells, PPARGC1A is associated
77 with anti-inflammatory [20,21] and anti-oxidant defence [22]; however, the impact of
78 exercise-induced inflammation and oxidative stress on PPARGC1A DNA methylation
79 is unknown. Epigenetic studies have linked a CpG site -260 bases from the
80 promoter of *PPARCG1A* with the regulation of mRNA expression. In skeletal muscle,
81 exercise can demethylate the *PPARGC1A* -260 CpG site which has been shown to
82 concurrently upregulate *PPARGC1A* mRNA expression [8,10,18]. Although well
83 characterised in skeletal muscle, the regulation of *PPARGC1A* expression in other
84 cells and tissues, including immune cells is poorly understood [19].

85

86 Exercise of sufficient intensity and duration can cause tissue injury and lead to a
87 systemic inflammatory response [14,23]. Increased circulating levels of the
88 inflammatory cytokines IL-6 and TNFa are strongly correlated with the progression of
89 sarcopenia and measures of physical performance [24,25]. Acute exercise can also
90 increase the production of reactive oxygen species, in both skeletal muscle and
91 immune cells [26], potentially leading to the development of oxidative stress and

92 damage to lipids, proteins and DNA [27,28]. Increases in markers of oxidative stress
93 and circulating levels of inflammatory cytokines, such as IL-6 and TNFa, have been
94 shown to alter the expression of DNA methyltransferases (DNMTs) [29–33] and
95 influence DNA methylation patterns [11,34]. DNA methylation of inflammatory
96 cytokines have been associated with various inflammatory diseases including *IL6*
97 with Rheumatoid Arthritis [35] and obesity [36]; *TNF* DNA methylation with type 2
98 diabetes [37] and Alzheimer's disease [38]. Despite increased circulating levels of
99 inflammatory cytokines post-exercise [14,23], the impact of exercise on the DNA
100 methylation of genes encoding inflammatory cytokines such as *IL6* and *TNF* remains
101 unknown.
102
103 There is the potential for the dietary supplementation of fatty acids (FAs) to prevent
104 the exercise-induced inflammation via the modulation of DNA methylation.
105 Supplementation of FAs, including omega-3 polyunsaturated FAs (n-3 PUFAs) and
106 extra virgin olive oil (EVOO), are consumed to reduce levels of inflammation [39,40],
107 however, the impact of these supplements on exercise-induced inflammation is
108 equivocal. Some studies have detected reductions in inflammation post-exercise with
109 FA supplementation [41,42], whereas, others have reported no change in
110 inflammation [43,44]. An emerging mechanism for the anti-inflammatory impact of FA
111 supplementation is via epigenetic modifications [45–48]. The supplementation of the
112 diet with krill oil, high in n-3 PUFAs, has been demonstrated to reduce PPARGC1A
113 mRNA expression and the change in mRNA expression was negatively correlated to
114 the change in plasma n-3 PUFAs [49]. Total n-3 PUFA content is negatively
115 correlated to both IL6 DNA methylation and IL-6 protein concentration [48].
116 EVOO is a commonly used control in exercise studies to assess the impact of n-3
117 PUFA; however, the supplementation of EVOO has also been reported to modify the
118 DNA methylation of genes associated with inflammation [50]. It remains to be
119 identified whether the supplementation of FAs have an epigenetic impact on
120 exercise-induced inflammation.
121
122 The present study investigated the impact of aerobic exercise on global and gene-
123 specific (*PPARGC1A*, *IL6* and *TNF*) DNA methylation and *DNMT* mRNA expression
124 in leukocytes of disease-free individuals. We also investigated whether these
125 relationships could be modified by the supplementation of FAs. The association

126 between physiological markers related to exercise performance, inflammation and
127 oxidative stress post exercise and DNA methylation were also investigated.
128
129

130 **Results**

131

132 **Global cytosine methylation and DNMT mRNA expression**

133 One-hour of cycling reduced global methylation, assessed by the Luminometric
134 Methylation Assay (LUMA; Figure 1A; Pre 79.2%; Post 78.7%, $p = 0.008$), and the
135 mRNA expression of both *DNMT3a* (Figure 1C; $p = 0.018$) and DNMT3b (Figure 1D;
136 $p = 0.046$). Supplementation of FAs did not alter global methylation or mRNA
137 expression of *DNMT3a* or *DNMT3b* (Figure 2; $p > 0.05$). While *DNMT1* mRNA
138 expression was unaffected by exercise, a significant interaction was identified
139 between supplement and trial ($p = 0.048$; Figure 2B) indicating differential effects on
140 mRNA expression with the two supplements. No correlation was detected between
141 global DNA methylation values and *DNMT* mRNA expression.
142

143 **Gene-specific DNA Methylation and mRNA expression**

144 ***PPARGC1A***

145 A reduction in *PPARGC1A* DNA methylation (Pre 6.9%; Post 6.3%, Figure 3A; $p <$
146 0.001) and an increase in mRNA expression (Figure 3B; $p < 0.001$) were detected
147 following exercise. The supplementation of FAs had no impact on *PPARGC1A* DNA
148 methylation or mRNA expression ($p > 0.05$). Moderate but non-significant negative
149 correlations were detected between *PPARGC1A* DNA methylation and *DNMT3a* and
150 *DNMT3b* mRNA expression (Figure 5).
151

152 ***IL6***

153 Despite an increase in IL-6 protein concentrations following exercise (Pre: $0.63 \pm$
154 0.24 pg/mL, Post: 3.78 ± 0.55 pg/mL; $p < 0.001$), there was no change in *IL6* DNA
155 methylation ($p > 0.05$) or mRNA expression ($p > 0.05$) following exercise. A
156 significant interaction was detected between supplement and trial for CpG3 (-1094)
157 indicating increased DNA methylation following n-3 PUFA and decreased

158 methylation following EVOO (Figure 4A; p = 0.038). A similar, non-significant (p =
159 0.080) trend was detected for IL6 mRNA expression following supplementation
160 (Figure 4B). A significant correlation was detected between the mean *IL6*
161 methylation across all CpG sites and *DNMT3b* mRNA expression (Figure 5, p =
162 0.007).
163

164 ***TNF***

165 Neither exercise or the supplementation of fatty acids altered *TNF* DNA methylation
166 or mRNA expression. Trends were identified between 3 *TNF* CpG sites and
167 differential methylation following supplementation (CpG2 p = 0.069; CpG3 p = 0.098;
168 CpG4 p = 0.067; CpGmean p = 0.077). *TNF* DNA methylation was negatively
169 correlated with *TNF* mRNA expression (Figure 5; p = 0.007). Moderate, however,
170 non-significant correlations were detected between both *IL6* and *DNMT3a* mRNA
171 expression, and TNF DNA methylation (Figure 5).
172
173

174 **Associations between DNA methylation and post-exercise physiology markers**

175 Figure 6 demonstrates the association between post-exercise DNA methylation and
176 physiological markers related to exercise, oxidative stress and inflammation. Prior to
177 FA supplementation, both *PPARGC1A* and *TNF* methylation post-exercise are
178 significantly correlated with Time Trial (TT) performance (Figure 6, $p < 0.05$).
179 Following the supplementation of n-3 PUFA and EVOO, correlations between TT
180 performance and both *PPARGC1A* and *TNF* DNA methylation are weakened and no
181 longer significant (Figure 6). A negative correlation was detected between peripheral
182 blood mononuclear cell (PBMC) protein carbonyl (PC) concentration, an intracellular
183 measure of oxidative stress, and both global and PPARGC1A methylation prior to
184 supplementation of FAs, however, no association was detected following n-3 PUFA
185 supplementation (Figure 6). The concentration of PC in serum, a systemic measure
186 of oxidative stress, was uncorrelated with DNA methylation at baseline, however,
187 following EVOO supplementation significant correlations existed between serum PCs
188 and both PPARGC1A and *TNF* DNA methylation (Figure 6). The only significant
189 correlation between DNA methylation and serum IL-6 concentration was a negative
190 correlation with global DNA methylation following n-3 PUFA supplementation (Figure
191 6).

192

193 **Discussion**

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

206

207 For the first time we report, global hypomethylation in leukocytes following an acute
208 bout of exercise. The only previous study to investigate the impact of acute exercise
209 in blood cells failed to detect any change in DNA methylation following correction for
210 multiple testing [11]. The results of the present study are in accordance with previous
211 reports of a net hypomethylation following chronic exercise training [2–7] and acute
212 bouts of exercise in plasma [51] and skeletal muscle [4,8]. Other studies have failed
213 to detect any change in global DNA methylation [52,53]; however, this can be
214 explained by a similar number of CpG sites increasing and decreasing in DNA
215 methylation [52]. It has also been demonstrated that exercise-induced
216 hypomethylation is retained during periods of detraining, allowing it to become
217 further hypomethylated following further training [4]. These data suggest that both
218 acute and chronic exercise is sufficient to alter DNA methylation patterns typically
219 resulting in hypomethylation.

220

221 The lack of concordance between a single bout of exercise and chronic exercise
222 training interventions indicates exercise may induce a transient state of
223 hypomethylation and repeated bouts of exercise reduce the impact of the stimulus.

224

225 In the present study, a 4-week supplementation of FAs did not influence global DNA
226 methylation. In contrast, a 6-month supplementation of n-3 PUFA decreased *LINE-1*
227 DNA methylation, a surrogate for global DNA methylation, in Alzheimer's patients
228 [54]. However, *LINE-1* methylation is increased in Alzheimer's patients compared to
229 healthy controls [55], therefore, the supplementation of n-3 PUFA in these individuals
230 may act to restore global DNA methylation to the normal level detected in healthy
231 individuals. The use of different surrogate measures of global methylation (LUMA vs
232 *LINE-1*) prevents the direct comparison between studies because of the different
233 region which these assays investigate. Two separate studies have indicated that the
234 methylation estimates provided by LINE-1 and LUMA are poorly correlated [56,57].
235
236 For the first time, post-exercise decreased methylation and concurrent increased
237 mRNA expression of *PPARGC1A* following a bout of aerobic exercise have been
238 detected in leukocytes. The results from the present study match previous reports of
239 aerobic exercise-induced hypomethylation in skeletal muscle [2,8,10] potentially
240 indicating a systemic impact of exercise on *PPARGC1A* DNA methylation. The
241 mRNA expression profile of skeletal muscle and PBMCs have been shown to be
242 highly associated following an 8-week supplementation of n-3 PUFAs [58]. Although
243 we do not find any association with *PPARGC1A* methylation / mRNA expression and
244 n-3 PUFA supplementation in the present study, the hypomethylation detected in the
245 present study is consistent with the impact of exercise in skeletal muscle providing
246 further evidence for blood-derived expression profiles to be used as a surrogate for
247 skeletal muscle.
248
249 The only previous report of *PPARGC1A* methylation from leukocytes failed to detect
250 an association with physical activity [59]. The lack of previous association could be
251 the result of the investigation of different CpG sites in the promoter region of
252 *PPARGC1A*. Alternatively, the discordance in these results could reflect the
253 heterogeneity in methylation pattern of immune cells [60]. Exercise increases the
254 number of circulating leukocytes, therefore, changes in methylation may be the result
255 of different proportions of leukocytes rather than a change in DNA methylation
256 patterns [61]. The present study has adjusted DNA methylation values to account for
257 the number of leukocytes (lymphocytes, neutrophils, monocytes, basophils and

258 eosinophils) [60], whereas, previous reports have failed to account for this critical
259 variable.
260
261 The positive correlation between leukocyte *PPARGC1A* methylation and exercise
262 performance indicates that increased DNA methylation may provide a performance
263 advantage. *PPARGC1A* is thought to upregulate mitochondrial biogenesis in
264 monocytes to induce a shift towards an anti-inflammatory phenotype [20,21] and
265 antioxidant defence in lymphocytes [22]. Although we did not find an association with
266 IL-6 protein concentration, a negative association was detected between
267 *PPARGC1A* DNA methylation and PC concentration indicating epigenetic control of
268 the antioxidant role of *PPARGC1A*. There is limited literature comparing
269 mitochondrial function in leukocytes and skeletal muscle following exercise;
270 however, the association between gait speed and mitochondrial function in both
271 skeletal muscle tissue and PBMCs provides a conserved mechanism between
272 mitochondrial function in skeletal muscle and blood-derived mitochondria [62].
273 Further evidence of a conserved mechanism is suggested with genes related to
274 mitochondrial structure and function found to be co-expressed in skeletal muscle and
275 neutrophils following aerobic exercise [63]. Future studies are required to detect if
276 the same phenotypic associations exist in skeletal muscle as detected in leukocytes
277 in the present study.
278
279 Aerobic exercise did not alter the DNA methylation or mRNA expression of either *IL6*
280 or *TNF*. The epigenetic impact of exercise on inflammatory cytokines is relatively
281 unknown, however, several studies have indicated a role for cytokine DNA
282 methylation in inflammatory disease [35–38]. Although no association between *TNF*
283 DNA methylation and mRNA expression was detected in the present study, n-3
284 PUFAs have previously been demonstrated to reverse the epigenetic changes
285 observed with inflammation in skeletal muscle cells. The administration of TNF
286 induced hypermethylation and decreased mRNA expression of MyoD [64], whereas
287 the supplementation of EPA dampens the impact of TNF in muscle and restores
288 MyoD mRNA expression [45]. Despite an increase in the circulating protein
289 concentration of IL-6 in the present study, the exercise bout may have not increased
290 TNFa protein concentration and induced an inflammatory response sufficient to
291 modify DNA methylation patterns of inflammatory cytokines. *TNF* hypermethylation is

292 reported in elderly individuals who maintained or increased their energy expenditure
293 by 500 kcal/wk over an 8-year period compared to those who decreased energy
294 expenditure over the same period [65]. The same *TNF* CpG sites as the present
295 study have previously been shown to negatively associate with mRNA expression,
296 plasma concentrations and measures of adiposity [66,67]. In the present study, a
297 significant negative correlation was detected between *TNF* DNA methylation post-
298 exercise and BMI, exercise performance and *TNF* mRNA expression. These data
299 suggest an acute bout of exercise may not regulate *TNF* DNA methylation, however,
300 the long-term benefits of regular exercise, such as reduced adiposity, may
301 subsequently increase *TNF* DNA methylation levels and as a result, reduce *TNF*
302 mRNA expression and the chronic low-grade inflammation levels associated with
303 increased adiposity.
304
305 Previously decreased methylation in a region ~600 bp upstream of the *IL6* promoter
306 has been associated with increased erythrocyte n-3 PUFA concentrations and
307 mRNA expression [48]. In the present study, the supplementation of EVOO and n-3
308 PUFA had contrasting effects on a single CpG (-1094) of *IL6* (increased methylation
309 following n-3 PUFA and decreased methylation with EVOO). The region ~1,000 bp
310 from upstream of was investigated in the present study because of previous
311 associations between DNA methylation and both inflammatory diseases [35,36] and
312 mRNA expression[35]. Conflicting results between studies may indicate that distinct
313 regions of the promoter regulate *IL6* expression differently. Supplementation of n-3
314 PUFA and OO have been shown to induce differential methylation of elongase and
315 desaturase enzymes which are responsible for the metabolism of FAs [68]. The
316 differential DNA methylation of these enzymes indicates the potential for n-3 PUFAs
317 to switch towards the production of less inflammatory eicosanoids. Although the DNA
318 methylation of desaturase and elongase enzymes have not been measured in the
319 present study, a switch towards n-3 PUFA derived eicosanoid production, such as 3-
320 series rather than 2-series prostaglandins, has been shown to reduce cytokine
321 expression [39] which is potentially indicated by the increased DNA methylation of
322 *IL6* following n-3 PUFA, but not EVOO, supplementation.
323
324 The impact of exercise and FA supplementation on *DNMT* mRNA expression was
325 investigated to identify whether changes in *DNMT* mRNA expression could be a

326 potential mechanism underlying modulated DNA methylation. *DNMT1* mRNA
327 expression was modulated by FA supplementation, whereas, exercise reduced the
328 expression of both *DNMT3a* and *DNMT3b*. This is the first demonstration of reduced
329 expression of *DNMT3a* following acute exercise, whereas, the reduction in *DNMT3b*
330 expression has previously been reported [32,69]. The inclusion of DNA methylation
331 assessment in the present study allows the confirmation that following a single bout
332 of aerobic exercise *DNMT* expression is decreased alongside decreases in global
333 and gene-specific DNA methylation. The only previous report of concurrent
334 assessment of exercise-induced *DNMT* expression and DNA methylation was
335 following an 8-week resistance training program [6]. The genome-wide method of
336 methylation does not identify a net increase or decrease in global methylation;
337 therefore, further studies are required to identify whether the modulation of *DNMT3b*
338 causes hypomethylation or if it is important in both hyper- and hypomethylation.
339
340 The present study detects contrasting effects of n-3 PUFA and EVOO
341 supplementation on *DNMT1* mRNA expression. There is a paucity of literature
342 surrounding the impact the FA supplementation and *DNMT* expression in humans,
343 whereas, animal models have associated supplementation of alpha-linolenic acid
344 supplementation, a n-3 PUFA, with changes in *DNMT* mRNA expression [70,71].
345 Interestingly, similar to the present study, no change in global DNA methylation was
346 detected alongside modulated *DNMT1* expression [70]. A change in global DNA
347 methylation potentially would not be expected with increased in *DNMT1* mRNA
348 expression because *DNMT1* functions to maintain DNA methylation. The impact of
349 EVOO on *DNMT* expression is unknown, however, EVOO contains phenolic
350 compounds, including decarboxymethyl oleuropein aglycone (DOA) [72], which
351 reduce DNMT activity via competitive inhibition [73]. The absence of a measure of
352 DNMT activity is a limitation of the present study, however, parallel changes in
353 DNMT mRNA expression and activity have previously been reported [74]. A measure
354 of activity could potentially explain the lack of association between altered *DNMT*
355 mRNA expression and modulated DNA methylation following supplementation which
356 should be considered in future studies.
357
358

359 While exercise and FA supplementation may directly influence *DNMT* expression,
360 these interventions may modulate *DNMT* expression by intermediary mechanisms.
361 The expression of several miRNAs, including miRNA-29 -130 and -148, are
362 associated with: DNMT expression [75–78], exercise [79] and FA supplementation
363 [80–82]. IL-6 protein levels have been reported to regulate *DNMT* mRNA expression
364 [31–33] via the modulation of miRNA [30]. The small increase in IL-6 protein
365 expression following exercise in the present study may be insufficient to modulate
366 *DNMT* expression explaining the lack of agreement with previous reports. Future
367 studies should use a bout of exercise with a greater inflammatory response, such as
368 eccentric exercise, to examine the effect of exercise-induced inflammation on *DNMT*
369 expression. The capability of exercise and n-3 PUFA supplementation to modify the
370 expression of the same miRNAs which control the expression of *DNMTs* suggests
371 miRNA expression could be one of the underlying mechanisms controlling DNA
372 methylation.
373
374 The use of a homogenous population of trained cyclists in the present study
375 potentially limits the generalisability of the results to other populations. Trained male
376 cyclists were selected as the population for the present study because they are the
377 most familiar with the exercise stimuli and we would expect this to reflect in the
378 smallest epigenetic response. Previously it has been demonstrated a single bout of
379 exercise was sufficient to reduce global DNA methylation in plasma of COPD
380 patients; however, following a training intervention the exercise bout was no longer
381 sufficient to reduce global DNA methylation [51]. Exercise training has previously
382 been demonstrated to alter DNA methylation patterns differently depending on family
383 history of diabetes [2]. Future studies should compare the impact of exercise in
384 trained athletes and sedentary individuals or a disease cohort to determine whether
385 exercise-induced alterations to the DNA methylome are contributors to health and
386 disease in diverse populations.
387
388 In conclusion, the present study highlights the impact of an acute bout of aerobic
389 exercise and the supplementation of FAs on DNA methylation and mRNA expression
390 in leukocytes of trained male cyclists. Alterations in the epigenetic control of these
391 genes are associated with physiological markers related to exercise performance
392 and inflammation / oxidative stress, however, a more extensive study is required to

393 confirm these associations. The observational nature of the present study prevents
394 the identification of the underlying mechanisms controlling altered DNA methylation
395 following exercise and FA supplementation, therefore, future mechanistic studies are
396 required to identify such mechanisms. Here we suggest that modulation of DNMT
397 mRNA expression may be one such mechanism for future research. Future studies
398 should compare multiple tissue types to examine whether exercise and
399 supplementation of FAs have systemic effects on DNA methylation.
400

401 ## Methods

402 ### Participants

403 Complete sets of data were available for eight participants whose characteristics are
404 described in table 1. Prior to participation, informed written consent was provided by
405 each participant. Participants were healthy, non-smokers with no history of metabolic
406 or cardiovascular disease. In the six-months prior to the study, participants had no
407 history of n-3 PUFA, anti-oxidant or anti-inflammatory supplementation. Participants
408 recorded their physical activity and maintained habitual diet throughout the study.
409 The experimental protocol was approved by the Loughborough University Ethics
410 Human Participants sub-committee and performed in accordance with the
411 Declaration of Helsinki 1975.
412

413 ### Study overview

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

418

419 ### Pre-test

420 Participants underwent anthropometric assessment for height, body mass and eight-
421 skinfold measurements prior to the start of the study. Maximal aerobic work rate
422 (Wmax) and maximal oxygen uptake ($\dot{V}O_{2max}$) were determined using a graded
423 exercise test on a Lode Excalibur Sport ergometer (Lode B.V, Netherlands). The

424 exercise test began with a warm-up period of 5-min cycling at 100 W. Workload then
425 increased by 50 W every 3-min until volitional fatigue (decrease in self-selected
426 cadence of 20 revs·min^{-1}). Expired air was collected in the final minute of each stage
427 to allow $\dot{V}O_{2max}$ determination using primary and secondary criteria [83]. Wmax was
428 calculated using the formula:

429 $$Wmax = Workload \div [(t/180) \times 50]$$

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

433

434 **Experimental trials**

435 Trials were conducted in the morning (7-9 am) following a 10-hour overnight fast.
436 Participants were asked to complete a 3-day food diary, refrain from strenuous
437 exercise and the consumption of alcohol or caffeine for the 24-hours prior to the trial.
438 The performance test consisted of 45-minutes cycling at 70% Wmax, followed by a
439 15-minute TT [84].

440

441 **Supplementation**

442 Both n-3 PUFA (Holland and Barrett, Warwickshire, UK) and EVOO (Puritan's Pride,
443 New York, USA) supplements were provided in capsule form. Participants were
444 instructed to take 6 capsules per day providing 5.7g of n-3 PUFA and 0.01g per day
445 of α-Tocopherol or 6 g per day of EVOO. The n-3 PUFA dose was chosen based on
446 previous findings showing the dose was sufficient to induce changes in the lipid
447 profile of human blood over four weeks [85,86]. Compliance of supplementation was
448 monitored by capsule counts.

449

450 **Analytic Procedures**

451 **Blood Sampling**

452 Venous blood was sampled via an intravenous catheter inserted into an antecubital
453 vein of the non-dominant arm for the collection of whole blood pre and immediately
454 post-exercise (Figure 7) for DNA methylation analysis, mRNA expression and a
455 whole blood cell count using the COULTER® Ac·T™ 5diff (Beckman Coulter, UK).
456 PBMCs were isolated from whole blood by density gradient centrifugation using
457 Ficoll-Paque Premium (GE healthcare, USA) according to manufacturer's
458 instructions. The resulting PBMC cell pellet was suspended in 200μl RIPA buffer for
459 analysis of protein carbonyls. Whole blood collected in vacutainers (Becton, Dickson
460 & Company, UK) that contained no anticoagulant was allowed to clot at room
461 temperature and centrifuged at 2800 rpm for 15 minutes for analysis of serum
462 protein carbonyls and IL-6.
463
464 **Nucleic acid isolation**
465 Genomic DNA (gDNA) was isolated from 2mL of whole blood using the QIAamp
466 DNA Blood Midi kit (Qiagen, Germany) according to the manufacturer's instructions.
467 RNA was isolated from whole blood collected in Tempus Blood RNA tubes using the
468 Tempus Spin RNA Isolation Kit (Applied Biosystems, USA) according to the
469 manufacturer's instructions. The concentration (mean ± SD) and purity (absorbance
470 ratio A_{260}/A_{280} ± SD) of isolated DNA and RNA were determined using a Nanodrop
471 2000 (ThermoScientific, USA). The mean concentration of isolated gDNA was
472 183.50 ± 54.48 ng/μL with a A_{260}/A_{280} ratio of 1.90 ± 0.02, whereas, RNA
473 concentration was 120.32 ± 41.02 ng/μL with an A_{260}/A_{280} ratio of 2.09 ± 0.02.
474 Following extraction, DNA and RNA were stored at -20 °C and -80 °C respectively.
475
476 **Luminometric Methylation Assay**
477 LUMA was used as a marker of global DNA methylation as previously described
478 [87], with minor adjustments. Briefly, two reactions containing 200 ng of gDNA were
479 set up per sample, one with the methylation-sensitive enzyme FastDigest HpaII and
480 one FastDigest MspI (Thermo Scientific, USA) and incubated for 20 min at 37 ˚C.
481 Following incubation, 13 μL of each reaction were mixed with annealing buffer and
482 added to a separate well of a Pyromark Q24 plate and analysed using a PyroMark
483 Q24 MDx system (Qiagen, Germany) with the following dispensation order:
484 ACTCGA. Peak heights were exported, and methylation percentage was calculated
485 using the following formula:

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

487

488 **Bisulfite pyrosequencing**

489 gDNA samples were bisulfite converted using the EpiTect Fast Bisulfite Conversion
490 Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR of bisulfite
491 converted DNA samples was performed using the PyroMark PCR Kit (Qiagen,
492 Germany) according to the manufacturer's instructions. For all assays, an initial
493 activation period of 15 min at 95˚C was followed by a 3-stage cycling process of
494 denaturation (95°C for 30s), annealing (56°C for 30 s) and extension (72°C for 30 s)
495 for 45 cycles. The PCR process was finished with a final extension period of 72°C for
496 10 min. Pyromark custom assay (Qiagen, Germany) genomic location, primer
497 sequences and the sequence to analyse are presented in Table 2. To confirm a
498 single PCR product, amplicons were analysed by gel electrophoresis and visualised
499 by ultraviolet trans-illuminator (BioRad, USA). The absence of PCR amplification of
500 non-bisulfite converted DNA confirmed the specificity of each assay for bisulfite
501 converted DNA. DNA methylation was assessed using a PyroMark Q48 Autoprep
502 system (Qiagen, Germany) using PyroMark Q48 Advanced CpG Reagents (Qiagen,
503 Germany). The nucleotide dispensation order was generated by entering the
504 sequence to analyse into the PyroMark Q48 Autoprep software version 2.4.2
505 (Qiagen, Germany). A non-CpG cytosine was included in the nucleotide dispensation
506 order to detect incomplete bisulfite conversion. The methylation at each CpG site
507 was determined using the PyroMark Q48 Autoprep software set in CpG mode. The
508 mean methylation of all CpG sites within the target region was determined using the
509 methylation at the individual CpG sites. Standards of known methylation percentages
510 (0%, 12.5%, 25%, 50%, 75%, 87.5%, 100%) were created using the EpiTect PCR
511 control DNA set (Qiagen, Germany) and underwent pyrosequencing analysis to
512 generate standard curves between the expected and observed methylation
513 percentage to check the assays for PCR bias. A high coefficient of determination (R^2
514 > 0.99) was determined for each assay indicating the absence of PCR bias.

515

516 **mRNA expression**

517 A minimum of 1 µg of RNA was reverse transcribed into complementary DNA
518 (cDNA) using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, USA)

519 according to the manufacturer's instructions and diluted to a concentration of 5 ng/µL
520 in double-distilled water. Relative mRNA expression was performed by quantitative
521 PCR (qPCR) for each gene of interest and normalised to the expression of *GAPDH*
522 using a Viia7 Real-Time PCR system (Applied Biosystems, USA). Each reaction
523 contained 5 µL of SybrGreen PrecisionPlus qPCR Master Mix (PrimerDesign, UK),
524 0.5 µL of forward and reverse primer (Table 3) and 4 µL of 5 ng/µL cDNA. All
525 samples were run in duplicate using the following cycling conditions: initial
526 denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for
527 60 s. Melt curves were visually inspected for a single peak indicating the generation
528 of a single product. The relative mRNA expression of the genes of interest were
529 calculated using the $2^{-(\Delta\Delta Ct)}$ formula; the pooled group mean pre-exercise Ct from the
530 initial trial was used as the control. The mean Ct value of *GAPDH* across all
531 participants and experimental conditions was 17.13 ± 0.41 with low variation of
532 2.40%. The efficiency of each mRNA expression assay was determined (Table 3)
533 using standard curves generated from a serial dilution of a cDNA sample. The
534 efficiency was calculated using the formula:
535 $E = ((10^{(-1/slope)}) - 1) \times 100$, where the slope is the gradient of the linear regression
536 fitted to the standard curve. The efficiency of each assay was between 90 and 105%
537 with a $R^2 > 0.99$.
538

539 **Interleukin-6 (IL-6)**

540 Serum IL-6 concentrations prior to and immediately post-exercise were determined
541 using high sensitivity enzyme immunoassay kits (R & D Systems, USA). Haematocrit
542 and haemoglobin were used to ascertain plasma volume changes that were used to
543 adjust serum IL-6 values [88].

544 **Protein Carbonyls (PC)**

545 PC was assessed by an in-house ELISA [89,90]. Serum samples, PBMC lysates and
546 standards were diluted in coating buffer (50mM sodium carbonate, pH = 9.2) to a
547 concentration of 0.05mg/mL using the bicinchoninic assay method. Protein carbonyls
548 groups were derivatised with 2, 4-dinitrophenylhydrazine (1mM, in 2M HCl) and
549 incubated with monoclonal mouse anti-DNP antibody (Sigma Aldrich, UK) and rat

550 anti-mouse IgE, conjugated to HRP (AbD Serotec, UK). Well absorbance was
551 measured at 490nm and the PC concentration determined by using absorbance
552 values of known PC standards made in our laboratory (1.28-5.20 nmol/mg protein).
553 PC concentration in PBMCs was adjusted for changes in protein concentration and
554 cell number (Beckman Coulter, UK) induced by acute exercise.

555 **Statistical Analysis**

556 All statistical analysis was performed using IBM SPSS Statistics software (SPSS
557 version 23). The data were assessed for normality by Shapiro-Wilk's test. The
558 composition of white blood cells from which the DNA is extracted is an important
559 consideration in DNA methylation research; therefore, all DNA methylation analysis
560 was conducted on cell heterogeneity adjusted values [60]. Analysis of mRNA
561 expression was performed on log fold change data. DNA methylation and mRNA
562 expression values were analysed using a 2 (supplement) x 2 (trial) x 2 (time)
563 repeated measures ANOVA. The impact of exercise is presented using the absolute
564 values (mean of all trials for each time point), whereas, the impact of
565 supplementation of FAs is presented as the relative change (Δ) between pre and
566 post supplementation trials (post supplementation – pre supplementation). Values
567 represented as mean $\pm$ 95% CI.

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

574

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578

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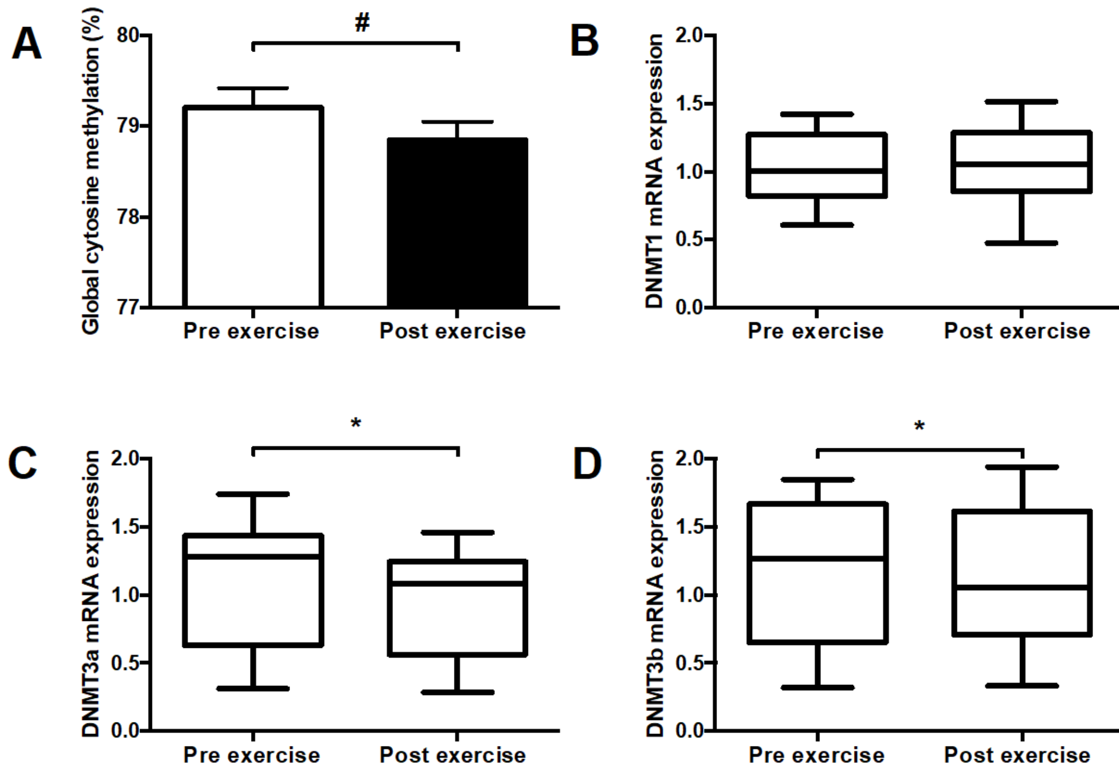

*Figure 1 – Effect of exercise on global DNA methylation (A) and mRNA expression of DNMT1 (B), DNMT3a (C) and DNMT3b (D). Data presented as the mean value of all trials for each time point. * p <0.05, # p <0.01*

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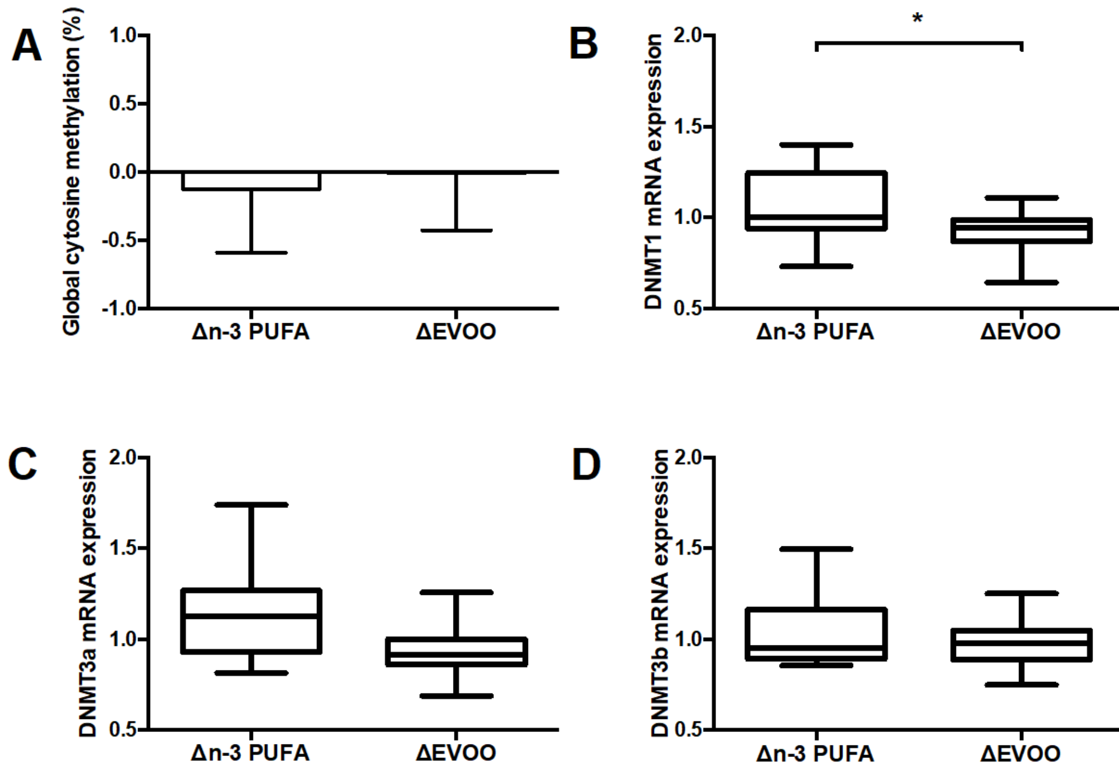

*Figure 2 - The impact of supplementation of n-3 PUFA and EVOO on global DNA methylation (A) and mRNA expression of DNMT1 (B), DNMT3a (C) and DNMT3b (D). Data presented as the relative change (Δ) between pre and post supplementation trials (post supplementation – pre supplementation) for each supplement.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

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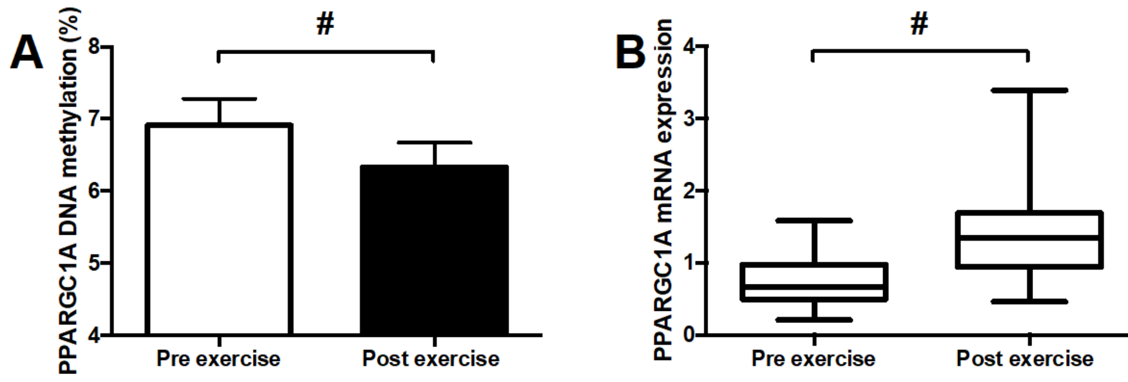

Figure 3 – Effect of exercise on DNA methylation of CpG-260 (A) and mRNA expression (B) of PPARGC1A. Data presented as the mean value of all trials for each time point. # $p < 0.01$

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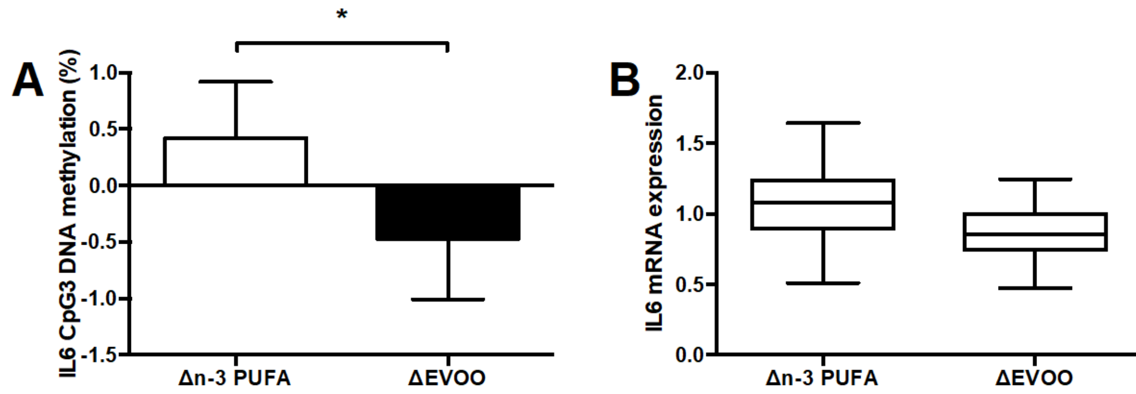

*Figure 4 – The impact of n-3 PUFA and EVOO supplementation on IL6 CpG3 DNA methylation (A) and IL6 mRNA expression (B). Data presented as the change (Δ) between pre and post supplementation trials (post supplementation – pre supplementation). n-3 PUFA, n-3 polyunsaturated fatty acid; EVOO, extra virgin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

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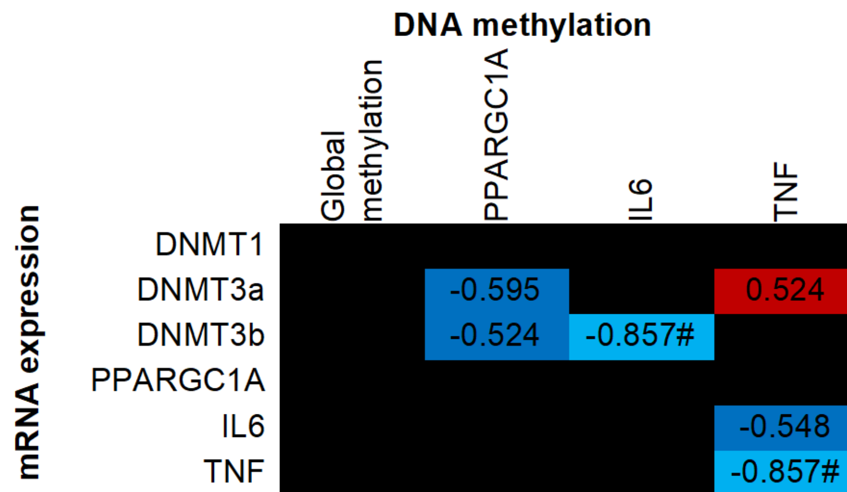

Correlation coefficient key:

*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. * $p < 0.05$, # $p < 0.01$*

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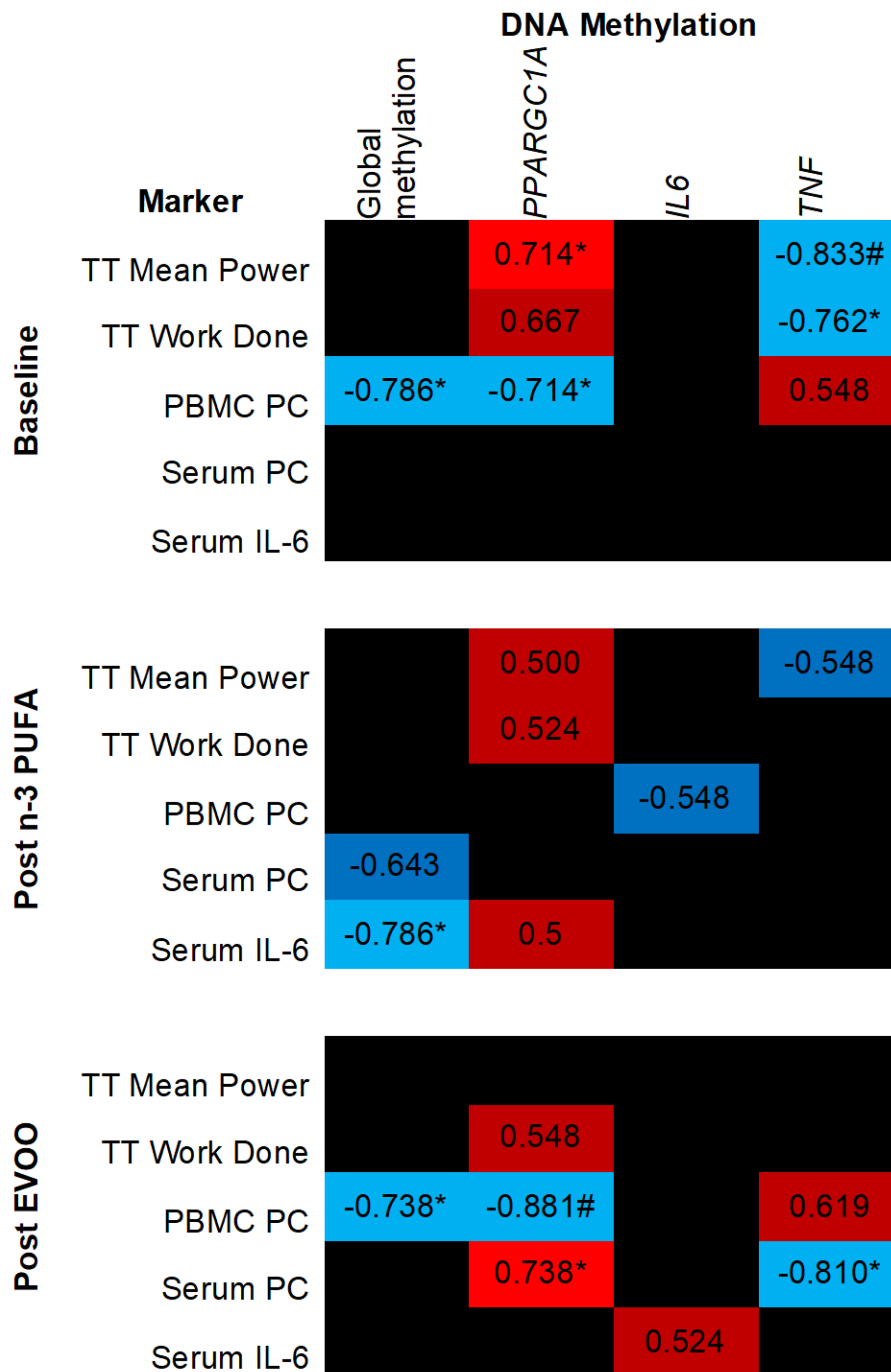

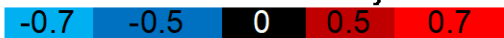

*Figure 6 – Spearman's Rho between post-exercise DNA methylation and physiological markers related to exercise performance, oxidative stress and inflammation. The mean of all CpG sites assessed for each gene has been used to provide an overall view of the region of interest. Blue indicates a negative 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.*

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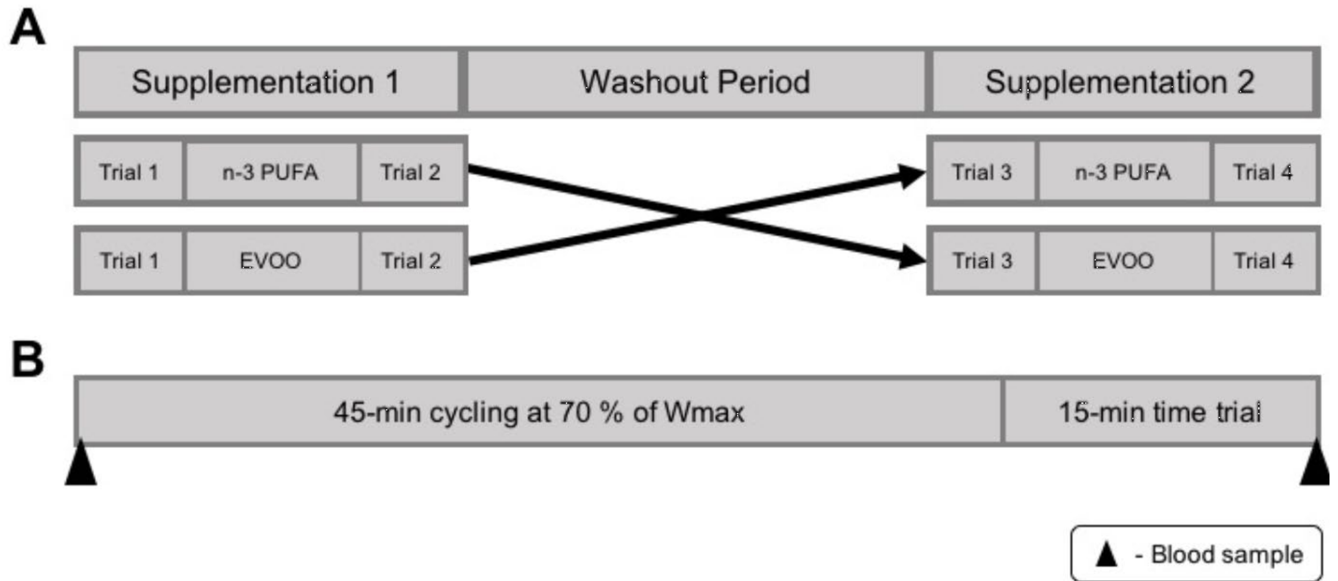

Figure 7 – Schematic representation of study outline (A) and trial day (B). n-3 PUFA, omega-3 polyunsaturated fatty acid; EVOO, extra virgin olive oil; Wmax, maximal aerobic work rate.

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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

962

*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 analyse 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 [chr4:23890308 - 23890372]	For:	5'-TGTAGGGGATTTTGGTTATTATATGGT-3'	1 (-260)
	Rev:	5'-biotin-ACCAACTTTAAATACCACAAACTCTA-3'	
	Seq:	5'-GGTTATTATATGGTTAGGGT-3'	
	Sequence to analyse:	TT**Y**GTTTAGAGTTTGTGGTATTTAAAGTT	
IL6 [chr7:22726051 - 22726198]	For:	5'-GGGAAGAGGGTTTTTGAATTAG-3'	6 (-1099, -1096, -1094, -1069, -1061 & -1057)
	Rev:	5'-biotin-CTCCCTCTCCCTATAAATCTTAATTTAA-3'	
	Seq:	5'-TTGAATTAGTTTGATTTAATAAGAA-3'	
	Sequence to analyse:	ATTTTTGGGTGT**Y**GA**Y**G**Y**GGAAGTAGATTTAGAGTTTAGAG T**Y**GTGTTTG**Y**GTT**Y**GTAGTTTTTTTTTAGTTTTTTTTGATTT	
TNF [chr6:31575730 - 31575816]	For:	5'-GGAAAGGATATTATGAGTATTGAAAGTATG-3'	4 (+197, +202, +214 & +222)
	Rev:	5'-biotin-CTAAAACCCCCTATCTTCTTAAA-3'	
	Seq:	5'-ATTATGAGTATTGAAAGTATGAT-3'	
	Sequence to analyse:	T**Y**GGGA**Y**GTGGAGTTGGT**Y**GAGGAGG**Y**GTTTTTTAAGAA GATAGGGGGGTTT	

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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'		

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