Articles in PresS. J Appl Physiol (December 24, 2014). doi:10.1152/japplphysiol.00878.2014

Total document: 10429

Main text: 5309

Abstract: 204

Number of Figures: 6

Number of Tables: 7

Supplemental Material: 1

Number of references: 68

Changes in the Leukocyte Methylome and its Effect on Cardiovascular Related Genes after Exercise

Running head: Exercise Changes the DNA Methylome

Joshua Denham[1], Brendan J O'Brien[2], Francine Z Marques[1], Fadi J Charchar[1]*

[1]Faculty of Science and Technology, Federation University Australia, Ballarat, Victoria, Australia

[2]Faculty of Health, Federation University Australia, Ballarat, Victoria, Australia

*Corresponding author: Fadi Charchar, University Drive, Ballarat, Victoria.

f.charchar@federation.edu.au, Ph: +613 5327 6098, Fax: +613 5327 9242.

J.D was involved in the study design, recruited, tested and trained subjects, performed laboratory experiments, analysed and interpreted data, and wrote the manuscript. B.J.O was involved in the study design, data interpretation and the writing of the manuscript. F.Z.M contributed to the study design, data interpretation and writing of the manuscript. F.J.C contributed to the study concept and design, interpreted data, revised the manuscript, funded and supervised the study.

Abstract

Physical exercise has proven cardiovascular benefits yet there is no clear understanding of the related molecular mechanisms leading to this. Here we determined the beneficial epigenetic effects of exercise after sprint interval training, a form of exercise known to improve cardio-metabolic health.

We quantified genome-wide leukocyte DNA methylation of 12 healthy young (18–24 y) men before and after four weeks (thrice weekly) of sprint interval training using the 450K BeadChip (Illumina) and validated gene expression changes in an extra seven subjects. Exercise increased subjects' cardio-respiratory fitness, maximal running performance and decreased low-density lipoprotein (LDL)-cholesterol concentration in conjunction with genome-wide DNA methylation changes. Notably, many CpG island and gene promoter regions were demethylated after exercise, indicating increased genome-wide transcriptional changes. Amongst genes with DNA methylation changes, epidermal growth factor (*EGF*), a ligand of the epidermal growth factor receptor known to be involved in cardiovascular disease, was demethylated and showed decreased mRNA expression. Additionally, we found that microRNAs, miR-21 and miR-210 gene DNA methylation were altered by exercise causing a cascade effect on the expression of the mature microRNA involved in cardiovascular function.

Our findings demonstrate that exercise alters DNA methylation in circulating blood cells in microRNA and protein coding genes associated with cardiovascular physiology.

Keywords: Epigenetics, sprint interval training, DNA methylation, cholesterol, miRNA

Introduction

Exercise training can prevent and attenuate symptoms of cardio-metabolic diseases, such as insulin resistance, elevated blood pressure (BP), excess adiposity and vascular impairment (18). The exact molecular mechanisms underpinning the health benefits gained from regular exercise training, however, are not known. DNA methylation affected by environmental factors modulates gene expression and may therefore mediate the health benefits gained from exercise training (19).

Recently, individuals involved in a six month moderate intensity exercise training program displayed genome-wide skeletal myocyte and adipocyte DNA methylation changes in genes associated with type 2 diabetes (49, 55). Additionally, older adult leukocyte DNA methylation increased in the pro-inflammatory *PYCARD* gene after six months of aerobic exercise training, to levels observed in their middle-aged peers (47). Although six months of moderate intensity aerobic exercise modulates the DNA methylome, it is unknown whether shorter training regimes impact DNA methylation. Moreover, while leukocytes are known to contribute to vascular disease (58), it is not known whether exercise is an environmental factor that regulates the leukocyte methylome and, in turn, contributes to improved vascular health.

Given the effects of acute exercise on DNA methylation are intensity-dependent (5), we aimed to determine whether leukocyte DNA methylation changes occur after short-term (four weeks) yet intense exercise training. We used sprint interval training as a form of exercise training because it rapidly improves vascular functioning (53, 54), insulin sensitivity (3), cardio-respiratory function and physical performance (reviewed in (25, 57)). We aimed to: 1) identify the effects of four weeks of thrice weekly exercise on genome-wide leukocyte DNA methylation in healthy young men; 2) establish whether specific DNA methylation changes occurred reciprocally with altered gene expression and; 3) identify the acute effect of maximal exercise on genes and microRNAs (miRNAs) related to cardiovascular disease. We

hypothesised that four weeks of exercise training would cause significant changes in DNA methylation in genes and miRNAs related to pathways involved in cardiovascular health.

Materials and Methods

Participants

Twenty-six healthy young men not already engaged in intense exercise training were recruited for this study. Participants were initially screened for any chronic diseases by health and physical activity readiness questionnaires. During the initial assessment, participants' height, weight and body-mass-index (BMI) were recorded. Resting BP was measured with the subjects seated using an electronic BP monitor (Microlife BP 3AQ1). Subjects' BP was measured after a 10 minute rest and was the average of two measurements broken up by a one minute rest period. Body fat percentage was estimated by summing seven skinfolds as described previously (31).

All subjects gave written informed consent and this study was approved by Federation University Australia's Human Research Ethics Committee.

Cardiopulmonary exercise testing and training

In order to assess cardiorespiratory fitness, participants completed one maximal oxygen consumption ($\dot{V}O_{2max}$) test, on the same day of the initial assessment and 48-96 hours following their final exercise session. Participants began training three days but no longer than one week after their $\dot{V}O_{2max}$ test and completed twelve training sessions (three per week) over four weeks. Participants completed two maximal treadmill exercise ($\dot{V}O_{2max}$) tests before and after exercise training. Before the $\dot{V}O_{2max}$ test participants were fitted with a two-

way breathing valve (Hans Rudolph, USA) and expired air was collected into an online metabolic system (Moxus, USA) for gas (O_2 and CO_2) analysis. The metabolic system was calibrated before each test using ambient air and gas of known composition. After a standardised five minute warm-up, the treadmill speed was increased one kilometre per hour every minute until the participant reached volitional exhaustion. Subject' $\dot{V}O_{2max}$ were determined as the highest oxygen consumption over one minute and was expressed as a relative value in $ml \cdot kg \cdot min^{-1}$ by dividing the $\dot{V}O_{2max}$ by body weight.

So as to restrict any potential DNA methylation changes to the exercise program, participants were requested not to deviate from their normal physical activity and recreational exercise training habits, as physical activity and exercise training habits influence leukocyte DNA methylation (19). Participants were also requested not to make changes to their normal diet, as dietary changes have been associated with DNA methylation alterations (46, 66). Participants had 48-72 hours recovery before the subsequent training session, to allow adequate recovery.

Before each exercise session, participants were instructed to perform a warm-up involving a brief low-intensity run (5 min), dynamic stretches and some short (20 m) high-intensity runs. Table 1 outlines the training schedule performed. Briefly, participants were required to complete three sprints at maximal intensity. To prevent overtraining, a two-up one-back training model was used. For example, during week one of training, participants completed three sprints on the first session, four sprints on the second session and five on the third and final session of week one. Participants then completed four sprints on the first session of week two (Table 1). The training was progressively overloaded until participants performed eight sprints on the final exercise session. Exercise sessions were monitored and supervised by an Accredited Exercise Physiologists (with Exercise and Sports Science Australia) to ensure the safety of participants. Training was performed on a university

athletics oval. Participants were requested to run at maximal intensity for each 30 sec effort. To ensure participants were running at maximal intensity, participants received constant verbal motivation. Participants began each effort at one end of the oval and individually ran around the oval until a whistle was blown to indicate the 30 sec had been completed. Participants were then given a 4 min rest, which involved a slow walk back to where they had started the test, followed by passive rest.

Blood processing

Participants donated a resting blood sample before and after their initial and final (after four weeks of exercise training) treadmill tests. Participants were asked to refrain from consuming alcohol or caffeinated beverages 24 hours before blood draw and were seated for approximately 30 minutes prior to their donations before and after the exercise intervention. Circulating blood was drawn from the antecubital vein into a serum separating tube and an EDTA tube with participant seated. All resting blood samples were collected from participants in the morning following an overnight fast. The final blood collection was obtained 48-96 hours after the final exercise session. Blood was temporarily stored on ice before further processing. The serum separating tube was left to clot at room temperature before centrifugation (3500 RPM) and was subsequently used to quantify blood lipid concentration using the CHOL2, TRIGL and HDLC3 reagents that were run on the Roche c701 instrument at Melbourne Pathology (Melbourne, Australia). All DNA and RNA were extracted on the same day, within three hours of blood draw to prevent possible *de nova* influences on DNA methylation and gene expression. DNA and RNA were extracted from whole blood stored in EDTA tubes using the PureLink Genomic DNA Mini Kit (Life Technologies) and miRNeasy Mini Kit (Qiagen), respectively, following the manufacturers' recommendations. The whole blood leukocytes were washed twice with the erythrocyte lysis wash buffer (included in the miRNeasy Mini Kit) to isolate leukocytes from plasma, serum,

platelets and lysed erythrocytes. Therefore, only whole blood leukocyte miRNA and gene expression were analysed in our study.

Genome-wide DNA methylation and EpiTYPER assay

While 19 participants completed the exercise intervention, whole-genome DNA methylation analysis was performed in 12 subjects at rest before and after the four week exercise intervention. The 450k BeadChip uses two types of Illumina chemistry to provide a comprehensive DNA methylation status of over 480,000 CpG sites spanning 99.9% of refseq genes. DNA methylation is represented as a β-value which corresponds to the percentage of methylation at a given CpG site. All samples quantified on the BeadChip passed the GenomeStudio Methylation Module's quality control procedure. To identify technical variation and increase the chances of detecting DNA methylation changes caused by exercise, raw β-values underwent Subset-quantile Within Array Normalization (SWAN) (44). Data was subsequently imported into and analysed using the bioinformatics software, Partek (Genomic Suite, version 6.6, Singapore). All β-values were log-transformed into M-values using the logit function ($\log^2(\beta/(1-\beta))$). M-values are a more valid alternative for detecting DNA methylation changes, as they eliminate the heteroscedasticity of higher- and lower-end β-values (22). Whereas M-values are more appropriate for analysing DNA methylation, β-values are more suitable for displaying biological relevance, as they correspond to the DNA methylation percentage of an individual CpG site. For this reason, M-values were converted back to β-values for all graphs and tables. Hierarchical clustering and pathway analysis was analysed using the software, Partek (Genomic Suite, version 6.6, Singapore). Due to the uneven CpG distributions between the two chemistries used on the Infinium HumanMethylation450 BeadChip (Illumina) – Infinium I (135,501) and Infinium II (350,076), we analysed these separately. Whole-genome DNA methylation was quantified using an Infinium HumanMethylation450 BeadChip (Illumina) according to the

manufacturer's guidelines and with the assistance of the Australian Genome Research Facility (Melbourne, Australia).

The EpiTYPER (Sequenom) was used to validate the DNA methylation change of the epidermal growth factor (*EGF*, cg12093976) and uracil-DNA glycosylase (*UNG*, cg20982606) genes, and these assays were performed by GeneWorks (Melbourne, Australia). Experiments were conducted according to the manufacturer's procedures. Briefly, 1µg of DNA was bisulfite converted and PCR amplified using the following primer sets: cg12093976: sense, aggaagagagAGTTATAATTTTTGGATTGGGGTTG and antisense, cagtaatacgactcactatagggagaaggctTAATTTAATTTTATCTCCATCCTTCCAA; cg20982606: sense, aggaagagagGATTATTTTGGAGTTGAGGAGGTAG and antisense, cagtaatacgactcactatagggagaaggctCCTTAAAAACCTATCCAAAAAACAA. Base-specific (C and T) cleavage and *in vitro* transcription was performed, followed by mass spectrometry (MALDI-TOF). Data was analysed using the EpiTYPER 1.2 software (Sequenom).

Gene and miRNA expression

The gene and miRNA expression of blood collected before and after initial and final treadmill exercise testing was assessed by qPCR using TaqMan assays (Life Technologies). RNA samples were reverse transcribed using the High Capacity Reverse Transcription Kit and the TaqMan MicroRNA Reverse Transcription Kit for gene and miRNAs, respectively, following the manufacturer's procedures (Life Technologies, Australia). Experiments on 384-well plates comprised of samples, endogenous positive and negative controls, all in duplicate were run on the ViiA 7 Real-Time PCR System (Life Technologies, Australia). Standard TaqMan assay procedures were followed and TaqMan assays used in the experiments are outlined in Table 2. The cycle threshold (Ct) of genes and miRNAs were compared to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the small-nucleolar RNA, *RNU44*, respectively. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (40) and the results were graphically represented as fold change. The cycling conditions were as

follows: a hold at 50° for 2 min and at 95° for 20 sec, followed by 40 cycles at 95° for 1 sec and 60° for 20 sec. As a quality control, duplicate samples greater than one Ct apart we re-run before being included in the analysis. All qPCR experiments were performed by the same researcher.

Statistical analyses

Participant phenotypes were assessed for normality using Kolmogorov-Smirnov and Shapiro-Wilks tests. Parametric data are expressed as mean ± standard deviation (SD) and non-parametric data are expressed by median (interquartile range). Non-parametric data was log-transformed before further analyses. While paired t-test was used to identify statistically significant changes to phenotypes, repeated measures ANOVA were used to show gene expression changes after four weeks of exercise training using the statistical software, IBM SPSS Statistics (version 21.0). Genome-wide DNA methylation changes in relation to CpG islands and gene regions were assessed using Chi^2 and Wilcoxon matched-pair signed ranked tests. DNA methylation changes caused by exercise training were determined using two-way ANOVA after β-values were log-transformed. In order to control for the discovery of false-positives, a false discovery rate (FDR) correction was applied to the whole-genome DNA methylation *P*-values by converting *P*-values to *q*-values. Whole genome data was analysed using the software, Partek Genomic Suite (version 6.6). Gene and miRNA expression were analysed using a two-way repeated measures ANOVA. The miRNA-mRNA targets were predicted using the miRWalk website (23), which includes predictions from the most commonly used prediction databases, and pathway analysis was conducted using Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) (29, 30). Statistical significance was determined as $P<0.05$.

Results

Exercise adherence

Of the 26 participants recruited into the intervention, 19 successfully completed the 12 exercise sessions. One participant withdrew due to a quadriceps musculoskeletal injury during training, one had a previous injury that was exacerbated by the exercise, another experienced an unrelated health concern and three stopped training for undisclosed reasons. The exercise adherence was approximately 73%.

Exercise improves cardiorespiratory health and fitness

Participants' phenotypes before and after exercise training are shown in Table 3. Exercise training increased cardiorespiratory fitness ($\dot{V}O_{2max}$) by 2.1ml·kg·min^{-1} (4.7%, *P*=0.03). Maximal running speed during $\dot{V}O_{2max}$ testing improved by 1km·hr^{-1} (4%, *P*=0.001) and LDL cholesterol was decreased after the intervention (-3.9%, *P*=0.047). Resting heart rate (-4%, *P*=0.05), diastolic BP (-4.3%, *P*=0.06) and total cholesterol (-3.3%, *P*=0.06) were reduced, while pulse pressure was increased (7.6%, *P*=0.07), all with borderline statistical significance.

*Whole-genome leukocyte DNA methylation changes a*fter exercise training

We analysed whole-genome DNA methylation using the 450 BeadChip (Illumina) to determine whether exercise altered the leukocyte methylome. The principle component analysis demonstrated separation between paired subject DNA methylation from samples taken before and after exercise training, indicating large changes to genome-wide DNA methylation (data not shown). DNA methylation changes occurred across the leukocyte genome in relation to gene regions and CpG islands (Figure 1). To gather an overview of

where these DNA methylation changes had occurred, we determined the average DNA methylation before and after exercise in relation to CpG islands and location in relation to the nearest gene. DNA methylation in relation to the nearest gene decreased in the promoter and intron regions for Infinium I and II assays, but increased in the 3' untranslated regions (UTR) for Inifinium I assay (Figure 2A, $P<0.0001$). There was a decrease in 5'UTR and exon DNA methylation in Infinium II assay (Figure 2A, $P<0.0001$). CpG island DNA methylation decreased in both Infinium I and II assays (Figure 2B, $P<0.0001$). Whereas Infinium II-assessed northern shore (N shore) DNA methylation deceased, southern shore (S shore) and northern shelf (N shelf) DNA methylation increased ($P<0.0001$, Figure 2B). Although the impact of specific regional DNA methylation changes are not completely understood, the CpG island and promoter region demethylation indicate an increase in transcriptional activity or gene un-silencing. Therefore, DNA methylation changes occurred across the leukocyte methylome after exercise training.

CpG methylation after exercise training

Out of the 485,577 CpG sites quantified for DNA methylation status on the Infinium HumanMethylation450 BeadChip (Illumina), exercise induced DNA methylation changes, ranging from 0.1–62.8%, at 205,987 sites relating to 32,445 transcripts after FDR ($q<0.05$). While 81,576 CpG sites relating to 16,256 transcripts became more methylated (Figure 2C, $q<0.05$), 124,411 CpG sites corresponding to 27,263 transcripts were de-methylated after exercise (Figure 2D, $q<0.05$).

In the search for biologically relevant changes to CpG site methylation, we identified CpG sites with a change of ≥5% after exercise ($q\leq0.05$) (Figure 3). Due to the large number of CpG sites (n=2,909) with a difference of ≥5%, we narrowed our focus to CpG sites (n=81) with a change of ≥10% ($q<0.005$, Table 4) and ≥20% after exercise ($q<0.005$, Table 5). Of the CpG sites that had large changes (≥20%) in DNA methylation after exercise, eight had

305 increased methylation and 11 had less methylation after exercise. Next, after applying a
306 stringent FDR (q<0.001) we used gene ontology to identify DNA methylation changes in
307 genes enriched for numerous cellular and molecular processes, including those involved in
308 metabolic activity, biological adhesion and antioxidant activity (Figure 4). Using pathway
309 analysis, we found that CpG sites altered by exercise (q<0.005) were those of genes related
310 to pathways important for cardiovascular physiology, including focal adhesion, calcium
311 signalling and MAPK signalling (q<0.05, Table 6). Therefore, differentially methylated CpG
312 sites after exercise are enriched for pathways crucial for cardiovascular health.
313
314 *DNA methylation validation*
315
316 DNA methylation within a gene promoter region is typically associated with decreased gene
317 expression, thus we focused on CpG sites with the largest and most statistically significant
318 DNA methylation changes within gene promoter regions (≥10% and $P<1.0\times10^{-5}$). To that
319 end, we analysed CpG sites within the *EGF* (cg12093976) and uracil-DNA glycosylase
320 (*UNG)* (cg20982606) promoter regions using the EpiTYPER (Sequenom). We were unable
321 to obtain meaningful data, due to the acquisition of peaks from additional fragments other
322 than the fragment of interest.
323
324 *Exercise-induced gene and miRNA expression*
325
326 We then aimed to identify whether a change in DNA methylation caused by exercise would
327 alter gene mRNA expression. We quantified the expression of genes that had a DNA
328 methylation change (>10%) that were most statistically significant (q<0.001) within the
329 promoter region or gene body (Figure 5A–F). We also quantified mature miR-21 and miR-
330 210 as their genes had a modest (1.3–4.2%) change in DNA methylation at multiple regions
331 across the gene (q<0.05, Table 7). These miRNAs are inversely correlated to
332 cardiorespiratory fitness and implicated in cardiovascular disease (11).

Gene expression changes after the initial $\dot{V}O_{2max}$ test, at rest after exercise training and after the second $\dot{V}O_{2max}$ test (after exercise training) are displayed in Figure 5. We were unable to successfully amplify insulin-like family member 3 (*IGFL3*) mRNA, suggesting that this gene is lowly expressed in leukocytes. Genes fitted into one of five categories; specifically, 1) genes that were responsive after acute exercise at the beginning of exercise training only (cell division cycle 20, *CDC20*); 2) genes that responded to acute exercise before and after exercise training (isthmin 1, *ISM1*); 3) genes that responded to acute exercise before and after exercise training and that had altered gene expression at rest after exercise training (*EGF*, *UNG* and miR-210); 4) those that were only altered at rest after exercise training (miR-21); and finally, 5) those that were unchanged by acute or chronic exercise (chromobox homolog 7, *CBX7* and synuclein α interacting protein, *SNCAIP*, data not shown). Interestingly, the demethylated (11.6% and 12.9%, respectively) *EGF* and *UNG* promoter was accompanied by a significant decrease and increase in mRNA expression, respectively (relative expression ± SEM, 10.75±1.68 to 8.25±0.95, $P<0.05$ and 1.72±0.14 to 2.12±0.15, $P=0.05$). These data suggest exercise training-induced DNA methylation changes within genes (including miRNA genes) cause changes to mRNA and mature miRNA levels (Figure 6).

miRNA-mRNA targets

Finally, we used miRWalk,(23) to predict mRNA targets of miR-21 and miR-210. We then used DAVID (29, 30) to identify pathways and diseases associated with mRNA molecules targeted by miR-21 and miR-210. Notably, miR-21 mRNA targets were enriched for pathways including MAPK signalling, Toll-like receptor signalling, apoptosis and fatty acid metabolism. Moreover, mRNA targets of miR-210 were enriched for genes relating to calcium signalling, B-cell receptor signalling and TGF-beta signalling pathways. While the mRNA targets of miR-21 were from genes associated with diseases, such as ischemic heart

360 disease (n=6), nephropathy (n=5) and coronary atherosclerosis (n=11), miR-210 mRNA
361 targets were from genes associated with focal dystonia (n=2). Collectively, these data
362 suggest a role for miR-21 and miR-210 in the positive cardiovascular adaptations associated
363 with short-term intense exercise training.
364
365 **Discussion**
366
367 Our study is the first, to our knowledge, to show genome-wide leukocyte DNA methylation
368 changes caused by short-term exercise in healthy young men. We found global and specific
369 leukocyte DNA methylation changes with concomitant changes to mRNA and miRNA
370 expression, in addition to favourable cardiovascular health adaptations after four weeks of
371 exercise training. Specifically, we observed DNA methylation changes to CpG islands and
372 gene promoter and gene bodies. We showed exercise training-induced demethylation of
373 cg12093976 and cg20982606, which decreased and increased the mRNA expression of the
374 *EGF* and *UNG* genes, respectively. Furthermore, exercise training-induced leukocyte DNA
375 methylation changes across miRNA genes (*MIR21* and *MIR210*) known to influence
376 cardiovascular physiology (20, 60), subsequently influencing their mature miRNA
377 expression.
378
379 Sprint interval training causes cardiopulmonary and metabolic adaptations similar to that of
380 traditional long duration aerobic training, but demands dramatically less time commitment
381 (10). Given a common barrier to engagement in exercise is lack of time, sprint interval
382 training may be an attractive alternative exercise training regime that improves health and
383 fitness. We demonstrated that four weeks of sprint interval training increased $\dot{V}O_{2max}$ and
384 reduced total and LDL cholesterol in healthy young men, thereby improving cardiorespiratory
385 fitness and blood lipid profile. We are, to our knowledge, the first to demonstrate that four
386 weeks of sprint interval training can significantly decrease LDL cholesterol in young men.
387 Although some have demonstrated eight weeks of sprint interval training, in the form of

388 running, is effective at reducing LDL and total cholesterol (56), other shorter (six week) sprint
389 interval training interventions have not reduced LDL cholesterol (27). Considering the role of
390 LDL cholesterol in blood vessel health (13, 15), sprint interval training may be a cheap and
391 effective short-term strategy to promote a favourable blood lipid profile.
392
393 Global leukocyte DNA methylation changes seem to be influenced by physical activity and
394 exercise training but data is equivocal. Although global leukocyte DNA methylation is
395 increased in middle-aged individuals performing moderate (65) or high amounts (63) of
396 physical activity in some studies, in others, global methylation is inversely (9, 41) related to
397 physical activity or unrelated (67). The DNA methylation of exon 1 of the *PYCARD* gene was
398 increased in older adults who engaged in six months of moderate intensity exercise, to
399 levels comparable to that of their younger peers (47). We are the first to analyse the
400 exercise training-induced changes to genome-wide leukocyte DNA methylation in healthy
401 subjects. Notably, we found a subtle decrease in leukocyte global methylation, consistent
402 with previous findings (9, 41). These data may indicate wide-spread transcriptional changes
403 to leukocytes. In particular, we found CpG island and gene body and promoter regions were,
404 on average, demethylated after exercise training, indicating the modulation of transcriptional
405 activity.
406
407 The exercise-induced changes to skeletal myocyte (49) and adipocyte (55) genome-wide
408 DNA methylation have been analysed previously. Strikingly, the exercise training-induced
409 changes to DNA methylation found in our study included CpG sites in genes involved in
410 pathways similar to that previously shown to be affected in skeletal myocytes after six
411 months of exercise training (49). For example, six months of moderate intensity exercise
412 altered skeletal muscle DNA methylation in genes related to MAPK signalling, progesterone-
413 mediated oocyte maturation, Wnt signalling, Melanogenesis, hedgehog signalling and
414 calcium signalling pathway (49) – pathways also modulated in leukocytes of individuals from
415 our study (Table 6). Furthermore, some of the mentioned pathways regulated by DNA

methylation changes caused by exercise may also be governed by miRNA-mediated mRNA regulation, as indicated by our miRNA pathway analysis.

Whereas skeletal myocytes and adipocyte DNA methylation changes are predominantly indicative of metabolic transcriptional changes caused by exercise, leukocytes changes could be used as biomarkers of systemic changes to health and fitness. Leukocytes circulate through the body and their gene expression mimics that of their internal and external environments, making them useful biomarkers (37). Likewise, the epigenetic landscapes are malleable to the internal (biological) and external environments (such as physical exercise) (7, 28). Therefore, leukocyte changes to epigenetic modifications could be relevant biomarkers of phenotype changes and in this case, biomarkers of the changes in cardiorespiratory fitness and accompanying health and performance adaptations (total and LDL cholesterol, and maximal running speed). Leukocytes are also implicated in the pathogenesis of atherosclerosis (58) and the DNA methylation changes observed in our study provide mechanistic insights into how exercise attenuates the risk of atherosclerosis.

Interestingly, exercise training caused DNA methylation changes with paralleled miRNA changes. DNA methylation can down-regulate gene expression by working in concert with other epigenetic modifications (e.g. Histone protein methylation and acetylation) and DNA binding proteins to compact chromatin and, in turn, inhibit transcription factor binding (17, 32, 33, 68). We identified that DNA methylation changes to *MIR21* and *MIR210* genes influenced the mature miR-21 and miR-210 expression. The microRNAs miR-21 and miR-210 are ubiquitously expressed hypoxamirs involved in cardiovascular diseases, inflammation and angiogenesis (14, 61). Indeed, miR-21 is up-regulated in atherosclerotic plaques (52) and acute coronary syndromes (64). The increased miR-21 expression, however, may be a protective response to the disease and acute coronary damage. For example, up-regulated miR-21 was protective against ischemia-induced cell apoptosis by down-regulating programmed cell death 4 (*PDCD4*) mRNA after myocardial infarction in rats

(21). Previous *in vitro* experiments showed miR-21 attenuates vascular smooth muscle cell apoptosis caused by reactive oxygen species (hydrogen peroxide, H_2O_2) through down-regulation of programmed cell death 4 (*PDCD4*) (38). Additionally, human endothelial cell miR-21 was augmented by shear stress and lead to decreased phosphatase and tensin homolog (*PTEN*)-mediated apoptosis, facilitated endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide (NO) production (62); an effect exerted by exercise training (26, 36). miR-21 is particularly responsive to exercise training as it is up-regulated after chronic exercise training in serum (4), spinal cord (39), cardiac (42) and skeletal myocytes (2) in mammals. Thus, exercise may maintain vascular integrity and prevent vascular disease through up-regulating miR-21 expression. This, in turn, contributes to modulation of miR-21 downstream mRNA targets involved in the eNOS pathway, oxidative stress and apoptosis.

miR-210 is induced by hypoxia and is up-regulated in numerous cardiovascular diseases, especially those associated with ischemia (20). Similar to miR-21, miR-210 is up-regulated in atherosclerotic plaques (52) and myocardial infarction (8), possibly by oxidative damage and cellular senescence. Reactive oxygen species (ROS) up-regulates miR-210 through phosphorylation of AKT, ERK1/2 and platelet-derived growth factor receptor β (34). miR-210 is also known to facilitate ROS production and to induce double-stranded DNA breaks in human cells undergoing replicative senescent (24). Interestingly, serum miR-21 and -210 were shown to be inversely correlated to $\dot{V}O_{2max}$ in a cohort of 100 healthy subjects (11). Our data also suggests miR-210 may be indicative of $\dot{V}O_{2max}$, as our participants exhibited elevated $\dot{V}O_{2max}$ with decreased leukocyte miR-210 expression after exercise training. We showed predicted targets of miR-21 are particularly enriched for mRNA from genes associated with cardiovascular pathology (ischemic heart disease and coronary atherosclerosis) that supports the concept but warrants further study. The impact of these exercise-induced miRNA changes on genes and biological implications is also left for future

research. Collectively, it is likely that miRNAs, miR-21 and miR-210, are exercise-responsive miRNAs that underpin the salubrious adaptations associated with exercise training.

Demethylation of *EGF* and *UNG* gene promoter regions attenuated and augmented the mRNA levels, respectively. Promoter demethylation is generally associated with gene activation (32), but not exclusively, as promoter demethylation does not always increase gene expression (12, 35, 45). Alternatively, the observed decrease to *EGF* mRNA expression could be due to other means of transcriptional regulation such as histone modifications, small non-coding RNA molecules or transcription factor activity. Importantly, EGF is a ligand for the epidermal growth factor-receptor (EGFR), which is hyperactive in atherosclerosis (43). The increased EGFR activity by elevated EGF may facilitate increased inflammation and blood vessel damage, leading to atherosclerosis (43). Acute exercise training increases peripheral but not central artery distensibility (53), which is an effect established after chronic exercise training (54). Moreover, exercise training increases and decreases muscle microvascular density and arterial stiffness, respectively (16). Therefore, the present study indicates that the attenuation of the EGFR-ligand, *EGF*, expression by promoter DNA methylation changes caused by exercise training could benefit vascular health. Similarly, the observed DNA methylation and gene expression change of *UNG* could serve as a mechanism for the demethylation of most genes analysed in the present study. Active DNA demethylation occurs via the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine by ten-eleven translocation (TET) proteins (1-3), followed by the active base-excision repair initiated by UNG (51, 59). Together, these data demonstrate the complex regulation of gene expression; where DNA methylation can directly regulate mRNA levels or, indirectly modulate gene expression through the modulation of miRNA expression subsequently influencing gene expression and cardiovascular phenotypes.

Although not measured, the numerous changes to DNA methylation observed in the present study could be a result of the altered DNA methyltransferase enzymes (DNMT1, 3A and 3B).

While DNMT1 is responsible for maintaining DNA methylation during mitosis, DNMT3A and -3B are responsible for *de novo* methylation changes (6). It is possible that exercise training-induced changes to activity of DNMT enzymes could have impacted the leukocyte methylome as there expression was altered in the hippocampi of mice after seven days of voluntary wheel running (1). Passive loss of DNA methylation during cell division is an alternate explanation for the DNA methylation changes observed in our study. This is, however, unlikely because of the short duration of study intervention period. The DNA methylation changes (up to 62.8%) caused by exercise training in our study could be explained by the intensity of exercise training. Previous work (5) revealed skeletal myocyte global and metabolic gene (*PPARGC1A*, *TFAM*, *PPARD*, *MEF2A*) DNA demethylation is intensity dependent, where higher intensity exercise elicited greater DNA demethylation. Moreover, the acute increase in reactive oxygen species and other metabolites may influence TET proteins and histone acetyltransferase and deacetylase activity to alter DNA methylation (50), but this remains to experimentally demonstrated.

Our study has some limitations. Blood leukocyte counts were not quantified before and after exercise training and it is possible that shifts in leukocyte subsets could be responsible for the change in leukocyte DNA methylation observed in our study. This is, however, unlikely as we quantified leukocyte DNA methylation from resting blood samples unaffected by the leukocytosis associated with acute exercise. Furthermore, there is no evidence to suggest leukocyte subsets change with chronic exercise training and a similar high-intensity exercise training study did not alter leukocyte subsets (48). Whist we did not include any experimental controls (who did not participate in any exercise for four weeks), data from our laboratory indicates that genome-wide leukocyte DNA methylation is stable over the course of eight weeks, as no changes were observed in a cohort of eight young men not engaging in any high-intensity aerobic exercise (unpublished data). We could not successfully validate the 450K DNA methylation results using EpiTYPER due to issues with the genomic regions being analysed. Exercise training was not performed in a laboratory setting and participants

exercise intensity was not directly monitored during the course of the intervention. Lastly, we cannot rule-out potential changes to participants' diet caused DNA methylation changes.

In conclusion, we have demonstrated that four weeks (249 min) of exercise training significantly alters the leukocyte methylome. These DNA methylation changes influenced miRNA and mRNA levels, improved cardiorespiratory fitness and enhanced the blood lipid profile of healthy, young men. These DNA methylation changes could be relevant biomarkers for monitoring adherence to exercise interventions and demonstrates a dynamic role for epigenetic regulation in the change to cardiovascular health and fitness caused by short-term exercise training.

Acknowledgements

We wish to express our gratitude to Dr Jack Harvey for his consultation on the statistical analyses used in the manuscript.

Grants

This work was supported by the Federation University Australia 'Self-sustaining Regions Research and Innovation Initiative', an Australian Government Collaborative Research Network (CRN). Joshua Denham is supported by an Australian Post-graduate Award scholarship. Dr Marques is supported by NHMRC (APP1052659) and National Heart Foundation (PF12M6785) co-shared Early Career Fellowships. Professor Charchar is supported by the Lew Carty Charitable fund and NHRMC.

Disclosures

None.

552 **References**

553

554 1. **Abel JL, and Rissman EF**. Running-induced epigenetic and gene
555 expression changes in the adolescent brain. *Int J Dev Neurosci* 31: 382-390, 2013.
556 2. **Aoi W, Naito Y, Mizushima K, Takanami Y, Kawai Y, Ichikawa H, and**
557 **Yoshikawa T**. The microRNA miR-696 regulates PGC-1{alpha} in mouse skeletal
558 muscle in response to physical activity. *Am J Physiol Endocrinol Metab* 298: E799-
559 806, 2010.
560 3. **Babraj JA, Vollaard NB, Keast C, Guppy FM, Cottrell G, and Timmons**
561 **JA**. Extremely short duration high intensity interval training substantially improves
562 insulin action in young healthy males. *BMC Endocr Disord* 9: 3, 2009.
563 4. **Baggish AL, Hale A, Weiner RB, Lewis GD, Systrom D, Wang F, Wang**
564 **TJ, and Chan SY**. Dynamic regulation of circulating microRNA during acute
565 exhaustive exercise and sustained aerobic exercise training. *J Physiol* 589: 3983-
566 3994, 2011.
567 5. **Barres R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, Caidahl K,**
568 **Krook A, O'Gorman DJ, and Zierath JR**. Acute exercise remodels promoter
569 methylation in human skeletal muscle. *Cell Metab* 15: 405-411, 2012.
570 6. **Bestor TH**. The DNA methyltransferases of mammals. *Hum Mol Genet* 9:
571 2395-2402, 2000.
572 7. **Bollati V, and Baccarelli A**. Environmental epigenetics. *Heredity (Edinb)*
573 105: 105-112, 2010.
574 8. **Bostjancic E, Zidar N, and Glavac D**. MicroRNA microarray expression
575 profiling in human myocardial infarction. *Dis Markers* 27: 255-268, 2009.
576 9. **Bryan AD, Magnan RE, Hooper AE, Harlaar N, and Hutchison KE**.
577 Physical activity and differential methylation of breast cancer genes assayed from
578 saliva: a preliminary investigation. *Ann Behav Med* 45: 89-98, 2013.

10. **Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL, and Gibala MJ**. Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol* 586: 151-160, 2008.

11. **Bye A, Rosjo H, Aspenes ST, Condorelli G, Omland T, and Wisloff U**. Circulating microRNAs and aerobic fitness--the HUNT-Study. *PLoS One* 8: e57496, 2013.

12. **Calvanese V, Fernandez AF, Urdinguio RG, Suarez-Alvarez B, Mangas C, Perez-Garcia V, Bueno C, Montes R, Ramos-Mejia V, Martinez-Camblor P, Ferrero C, Assenov Y, Bock C, Menendez P, Carrera AC, Lopez-Larrea C, and Fraga MF**. A promoter DNA demethylation landscape of human hematopoietic differentiation. *Nucleic Acids Res* 40: 116-131, 2012.

13. **Chapman MJ, Ginsberg HN, Amarenco P, Andreotti F, Boren J, Catapano AL, Descamps OS, Fisher E, Kovanen PT, Kuivenhoven JA, Lesnik P, Masana L, Nordestgaard BG, Ray KK, Reiner Z, Taskinen MR, Tokgozoglu L, Tybjaerg-Hansen A, Watts GF, and European Atherosclerosis Society Consensus P**. Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. *Eur Heart J* 32: 1345-1361, 2011.

14. **Chen KC, and Juo SH**. MicroRNAs in atherosclerosis. *Kaohsiung J Med Sci* 28: 631-640, 2012.

15. **Cholesterol Treatment Trialists C, Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R, and Baigent C**. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *Lancet* 380: 581-590, 2012.

16. **Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe AM, Barker TA, Tipton KD, and Wagenmakers AJ**. Sprint interval and endurance training are

607 equally effective in increasing muscle microvascular density and eNOS content in
608 sedentary males. *J Physiol* 591: 641-656, 2013.
609 17. **Comb M, and Goodman HM**. CpG methylation inhibits proenkephalin gene
610 expression and binding of the transcription factor AP-2. *Nucleic Acids Res* 18: 3975-
611 3982, 1990.
612 18. **Dangardt FJ, McKenna WJ, Luscher TF, and Deanfield JE**. Exercise:
613 friend or foe? *Nat Rev Cardiol* 10: 495-507, 2013.
614 19. **Denham J, Marques FZ, O'Brien BJ, and Charchar FJ**. Exercise: putting
615 action into our epigenome. *Sports Med* 44: 189-209, 2014.
616 20. **Devlin C, Greco S, Martelli F, and Ivan M**. miR-210: More than a silent
617 player in hypoxia. *IUBMB Life* 63: 94-100, 2011.
618 21. **Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, Wang D, Krall TJ, Delphin**
619 **ES, and Zhang C**. MicroRNA expression signature and the role of microRNA-21 in
620 the early phase of acute myocardial infarction. *J Biol Chem* 284: 29514-29525, 2009.
621 22. **Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, and Lin SM**.
622 Comparison of Beta-value and M-value methods for quantifying methylation levels by
623 microarray analysis. *BMC Bioinformatics* 11: 587, 2010.
624 23. **Dweep H, Sticht C, Pandey P, and Gretz N**. miRWalk--database: prediction
625 of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed*
626 *Inform* 44: 839-847, 2011.
627 24. **Faraonio R, Salerno P, Passaro F, Sedia C, Iaccio A, Bellelli R, Nappi TC,**
628 **Comegna M, Romano S, Salvatore G, Santoro M, and Cimino F**. A set of miRNAs
629 participates in the cellular senescence program in human diploid fibroblasts. *Cell*
630 *Death Differ* 19: 713-721, 2012.
631 25. **Gist NH, Fedewa MV, Dishman RK, and Cureton KJ**. Sprint interval training
632 effects on aerobic capacity: a systematic review and meta-analysis. *Sports Med* 44:
633 269-279, 2014.

26. **Hambrecht R, Adams V, Erbs S, Linke A, Krankel N, Shu Y, Baither Y, Gielen S, Thiele H, Gummert JF, Mohr FW, and Schuler G**. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation* 107: 3152-3158, 2003.

27. **Hazell TJ, Hamilton CD, Olver TD, and Lemon PW**. Run sprint interval training induces fat loss in women. *Applied Physiology, Nutrition, and Metabolism* 2014.

28. **Heyn H, and Esteller M**. DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet* 13: 679-692, 2012.

29. **Huang da W, Sherman BT, and Lempicki RA**. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37: 1-13, 2009.

30. **Huang da W, Sherman BT, and Lempicki RA**. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4: 44-57, 2009.

31. **Jackson AS, and Pollock ML**. Generalized equations for predicting body density of men. *Br J Nutr* 40: 497-504, 1978.

32. **Jones PA**. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 13: 484-492, 2012.

33. **Kass SU, Landsberger N, and Wolffe AP**. DNA methylation directs a time-dependent repression of transcription initiation. *Curr Biol* 7: 157-165, 1997.

34. **Kim JH, Park SG, Song SY, Kim JK, and Sung JH**. Reactive oxygen species-responsive miR-210 regulates proliferation and migration of adipose-derived stem cells via PTPN2. *Cell Death Dis* 4: e588, 2013.

35. **Klug M, Heinz S, Gebhard C, Schwarzfischer L, Krause SW, Andreesen R, and Rehli M**. Active DNA demethylation in human postmitotic cells correlates with

activating histone modifications, but not transcription levels. *Genome Biol* 11: R63, 2010.

36. **Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, and Nickenig G**. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 109: 220-226, 2004.

37. **Liew CC, Ma J, Tang HC, Zheng R, and Dempsey AA**. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. *J Lab Clin Med* 147: 126-132, 2006.

38. **Lin Y, Liu X, Cheng Y, Yang J, Huo Y, and Zhang C**. Involvement of MicroRNAs in hydrogen peroxide-mediated gene regulation and cellular injury response in vascular smooth muscle cells. *J Biol Chem* 284: 7903-7913, 2009.

39. **Liu G, Detloff MR, Miller KN, Santi L, and Houle JD**. Exercise modulates microRNAs that affect the PTEN/mTOR pathway in rats after spinal cord injury. *Exp Neurol* 233: 447-456, 2012.

40. **Livak KJ, and Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.

41. **Luttropp K, Nordfors L, Ekstrom TJ, and Lind L**. Physical activity is associated with decreased global DNA methylation in Swedish older individuals. *Scand J Clin Lab Invest* 73: 184-185, 2013.

42. **Ma Z, Qi J, Meng S, Wen B, and Zhang J**. Swimming exercise training-induced left ventricular hypertrophy involves microRNAs and synergistic regulation of the PI3K/AKT/mTOR signaling pathway. *Eur J Appl Physiol* 113: 2473-2486, 2013.

43. **Makki N, Thiel KW, and Miller FJ, Jr.** The epidermal growth factor receptor and its ligands in cardiovascular disease. *Int J Mol Sci* 14: 20597-20613, 2013.

687 44. **Maksimovic J, Gordon L, and Oshlack A**. SWAN: Subset-quantile within
688 array normalization for illumina infinium HumanMethylation450 BeadChips. *Genome*
689 *Biol* 13: R44, 2012.
690 45. **Martin-Subero JI, Kreuz M, Bibikova M, Bentink S, Ammerpohl O,**
691 **Wickham-Garcia E, Rosolowski M, Richter J, Lopez-Serra L, Ballestar E, Berger**
692 **H, Agirre X, Bernd HW, Calvanese V, Cogliatti SB, Drexler HG, Fan JB, Fraga**
693 **MF, Hansmann ML, Hummel M, Klapper W, Korn B, Kuppers R, Macleod RA,**
694 **Moller P, Ott G, Pott C, Prosper F, Rosenwald A, Schwaenen C, Schubeler D,**
695 **Seifert M, Sturzenhofecker B, Weber M, Wessendorf S, Loeffler M, Trumper L,**
696 **Stein H, Spang R, Esteller M, Barker D, Hasenclever D, Siebert R, and**
697 **Molecular Mechanisms in Malignant Lymphomas Network Project of the**
698 **Deutsche K**. New insights into the biology and origin of mature aggressive B-cell
699 lymphomas by combined epigenomic, genomic, and transcriptional profiling. *Blood*
700 113: 2488-2497, 2009.
701 46. **Milagro FI, Campion J, Cordero P, Goyenechea E, Gomez-Uriz AM,**
702 **Abete I, Zulet MA, and Martinez JA**. A dual epigenomic approach for the search of
703 obesity biomarkers: DNA methylation in relation to diet-induced weight loss. *FASEB*
704 *J* 25: 1378-1389, 2011.
705 47. **Nakajima K, Takeoka M, Mori M, Hashimoto S, Sakurai A, Nose H,**
706 **Higuchi K, Itano N, Shiohara M, Oh T, and Taniguchi S**. Exercise effects on
707 methylation of ASC gene. *Int J Sports Med* 31: 671-675, 2010.
708 48. **Ndon JA, Snyder AC, Foster C, and Wehrenberg WB**. Effects of chronic
709 intense exercise training on the leukocyte response to acute exercise. *Int J Sports*
710 *Med* 13: 176-182, 1992.
711 49. **Nitert MD, Dayeh T, Volkov P, Elgzyri T, Hall E, Nilsson E, Yang BT, Lang**
712 **S, Parikh H, Wessman Y, Weishaupt H, Attema J, Abels M, Wierup N, Almgren**
713 **P, Jansson PA, Ronn T, Hansson O, Eriksson KF, Groop L, and Ling C**. Impact

714 of an exercise intervention on DNA methylation in skeletal muscle from first-degree
715 relatives of patients with type 2 diabetes. *Diabetes* 61: 3322-3332, 2012.
716 50. **Pareja-Galeano H, Sanchis-Gomar F, and Garcia-Gimenez JL**. Physical
717 exercise and epigenetic modulation: elucidating intricate mechanisms. *Sports Med*
718 44: 429-436, 2014.
719 51. **Pastor WA, Aravind L, and Rao A**. TETonic shift: biological roles of TET
720 proteins in DNA demethylation and transcription. *Nat Rev Mol Cell Biol* 14: 341-356,
721 2013.
722 52. **Raitoharju E, Lyytikainen LP, Levula M, Oksala N, Mennander A, Tarkka**
723 **M, Klopp N, Illig T, Kahonen M, Karhunen PJ, Laaksonen R, and Lehtimaki T**.
724 miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human
725 atherosclerotic plaques in the Tampere Vascular Study. *Atherosclerosis* 219: 211-
726 217, 2011.
727 53. **Rakobowchuk M, Stuckey MI, Millar PJ, Gurr L, and Macdonald MJ**.
728 Effect of acute sprint interval exercise on central and peripheral artery distensibility in
729 young healthy males. *Eur J Appl Physiol* 105: 787-795, 2009.
730 54. **Rakobowchuk M, Tanguay S, Burgomaster KA, Howarth KR, Gibala MJ,**
731 **and MacDonald MJ**. Sprint interval and traditional endurance training induce similar
732 improvements in peripheral arterial stiffness and flow-mediated dilation in healthy
733 humans. *Am J Physiol Regul Integr Comp Physiol* 295: R236-242, 2008.
734 55. **Ronn T, Volkov P, Davegardh C, Dayeh T, Hall E, Olsson AH, Nilsson E,**
735 **Tornberg A, Dekker Nitert M, Eriksson KF, Jones HA, Groop L, and Ling C**. A six
736 months exercise intervention influences the genome-wide DNA methylation pattern in
737 human adipose tissue. *PLoS Genet* 9: e1003572, 2013.
738 56. **Sandvei M, Jeppesen PB, Stoen L, Litleskare S, Johansen E, Stensrud T,**
739 **Enoksen E, Hautala A, Martinmaki K, Kinnunen H, Tulppo M, and Jensen J**.
740 Sprint interval running increases insulin sensitivity in young healthy subjects. *Arch*
741 *Physiol Biochem* 118: 139-147, 2012.

742 57. **Sloth M, Sloth D, Overgaard K, and Dalgas U**. Effects of sprint interval
743 training on VO2max and aerobic exercise performance: A systematic review and
744 meta-analysis. *Scand J Med Sci Sports* 23: e341-352, 2013.
745 58. **Swirski FK, and Nahrendorf M**. Leukocyte behavior in atherosclerosis,
746 myocardial infarction, and heart failure. *Science* 339: 161-166, 2013.
747 59. **Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y,**
748 **Agarwal S, Iyer LM, Liu DR, Aravind L, and Rao A**. Conversion of 5-
749 methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner
750 TET1. *Science* 324: 930-935, 2009.
751 60. **Urbich C, Kuehbacher A, and Dimmeler S**. Role of microRNAs in vascular
752 diseases, inflammation, and angiogenesis. *Cardiovasc Res* 79: 581-588, 2008.
753 61. **Weber C, Schober A, and Zernecke A**. MicroRNAs in arterial remodelling,
754 inflammation and atherosclerosis. *Curr Drug Targets* 11: 950-956, 2010.
755 62. **Weber M, Baker MB, Moore JP, and Searles CD**. MiR-21 is induced in
756 endothelial cells by shear stress and modulates apoptosis and eNOS activity.
757 *Biochem Biophys Res Commun* 393: 643-648, 2010.
758 63. **White AJ, Sandler DP, Bolick SC, Xu Z, Taylor JA, and DeRoo LA**.
759 Recreational and household physical activity at different time points and DNA global
760 methylation. *Eur J Cancer* 49: 2199-2206, 2013.
761 64. **Yao R, Ma Y, Du Y, Liao M, Li H, Liang W, Yuan J, Ma Z, Yu X, Xiao H,**
762 **and Liao Y**. The altered expression of inflammation-related microRNAs with
763 microRNA-155 expression correlates with Th17 differentiation in patients with acute
764 coronary syndrome. *Cell Mol Immunol* 8: 486-495, 2011.
765 65. **Zhang FF, Cardarelli R, Carroll J, Zhang S, Fulda KG, Gonzalez K,**
766 **Vishwanatha JK, Morabia A, and Santella RM**. Physical activity and global
767 genomic DNA methylation in a cancer-free population. *Epigenetics* 6: 293-299, 2011.
768 66. **Zhang FF, Morabia A, Carroll J, Gonzalez K, Fulda K, Kaur M,**
769 **Vishwanatha JK, Santella RM, and Cardarelli R**. Dietary patterns are associated

770 with levels of global genomic DNA methylation in a cancer-free population. *J Nutr*
771 141: 1165-1171, 2011.
772 67. **Zhang FF, Santella RM, Wolff M, Kappil MA, Markowitz SB, and Morabia**
773 **A**. White blood cell global methylation and IL-6 promoter methylation in association
774 with diet and lifestyle risk factors in a cancer-free population. *Epigenetics* 7: 606-614,
775 2012.
776 68. **Zhao Q, Rank G, Tan YT, Li H, Moritz RL, Simpson RJ, Cerruti L, Curtis**
777 **DJ, Patel DJ, Allis CD, Cunningham JM, and Jane SM**. PRMT5-mediated
778 methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA
779 methylation in gene silencing. *Nat Struct Mol Biol* 16: 304-311, 2009.
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797

798 **Figure Captions**

799 Figure 1 DNA methylation changes in relation to CpG islands (A and B) and the
800 nearest gene (C and D), respectively. Data are from Chi^2 tests.
801
802 Figure 2. Whole-genome DNA methylation changes caused by exercise. Genome
803 wide DNA methylation across all 485,577 CpG sites analysed on the Infinium
804 HumanMethylation450 BeadChip (Illumina, Australia) were compared before and
805 after exercise in relation to A) nearest gene and B) CpG island. Data are from
806 Wilcoxon matched-pair signed ranked test and are expressed as mean ± SEM.
807 Number of CpG sites with C) increased methylation and D) decreased methylation,
808 with magnitude of DNA methylation change after exercise (q<0.05).
809 Legend: ***P<0.0001; N: northern; S: southern; 3'UTR: 3-prime un-translated region;
810 5'UTR: 5-prime un-translated region; Promoter: gene promoter region (1–1500 bases
811 upstream of the transcription start site).
812
813 Figure 3. Hierarchical clustering of CpG methylation before and after exercise
814 training.
815 The CpG methylation status is indicated by green (low methylation) and red (high
816 methylation) in 12 subjects pre (grey bar) and post (blue bar) exercise training. Each
817 branch of the array tree on the left of the grey and blue bars are subjects and are
818 separated before and after the exercise training. The array tree at the top of the
819 hierarchical cluster indicates clusters of CpG sites modified by exercise.
820 Data are from CpG sites with a fold-change of >5% (q<0.001).
821
822 Figure 4. Gene ontology for genes with CpG DNA methylation changes after
823 exercise. Gene ontology is based on CpG sites with altered DNA methylation
824 (q<0.001) after exercise training. Gene ontology enrichment scores are given and

825 organised by A) biological processes; B) cellular components; and C) molecular
826 functions.
827
828 Figure 5. Gene and miRNA expression changes caused by exercise. Data are from
829 two-way repeated-measures ANOVA and are expressed as fold change of genes
830 and mature miRNA expression (mean ± SEM). A significant difference between basal
831 and other time-points is indicated by asterisks.
832 Legend: *P<0.05; **P<0.01; ***P<0.001 vs basal.
833
834 Figure 6. Schematic of DNA methylation regulation on cardiovascular physiology.
835 Exercise training, in the form of sprint interval training (SIT), influences the leukocyte
836 DNA methylome. This, in turn, modulates the expression (mRNA levels) of protein-
837 coding genes (*EGF* and *UNG*) and also microRNAs (miR-21 and -210). MicroRNAs,
838 miR-21 and miR-210 would subsequently regulate other protein-coding genes to
839 inevitably influence cardiovascular physiology and enhance cardiovascular health
840 and performance.
841
842
843
844
845
846
847
848
849
850
851
852

853

854 **Tables**

855 Table 1. Description of the four week exercise program.

Week	Session #	Training load*	Training sprint time (min)	Total session time (min)
1	1	3 sprints	1.3	9.5
	2	4 sprints	2	14
	3	5 sprints	2.5	18.5
2	4	4 sprints	2	14
	5	5 sprints	2.5	18.5
	6	6 sprints	3	23
3	7	5 sprints	2.5	18.5
	8	6 sprints	3	23
	9	7 sprints	3.5	27.5
4	10	6 sprints	3	23
	11	7 sprints	3.5	27.5
	12	8 sprints	4	32
	Total Time	66	33	249

856 Legend: # number; * all sprints were completed at maximum intensity and separated

857 by four minutes of passive recovery.

858

859

860

861

862

863

864

865

866 Table 2. TaqMan Assay information.

Gene/microRNA	**TaqMan ID**
CBX7	Hs00545603_m1
CDC20	Hs00426680_mH
EGF	Hs01099999_m1
GAPDH (mRNA reference gene)	Hs02786624_g1
IGFL3	Hs00419511_g1
ISM1	Hs01382748_m1
SNCAIP	Hs00917423_m1
UNG	Hs01037093_m1
hsa-miR-21	000397
hsa-miR-210	000512
RNU44 (miRNA reference gene)	001094

867

868

869

870

871

872

873

874

875

876

877

878

879

880

881

882 Table 3. Participant phenotypes before and after exercise training.

Phenotype	Before exercise Mean±SD	After exercise Mean±SD	% Change	*P*-value
		n=19		
Age (years)		21.1±2.7		
Height (cm)		180.6±7.4		
Anthropometric				
Weight (kg)	78.1±9.4	78.1±9.8	-0.03±2.3	0.99
BMI (kg/m^2)	23.9±2.3	23.9±2.4	-0.2±2.4	0.68
Waist (cm)	79.2±5.3	78.9±4.8	-0.4±2.5	0.50
Hip (cm)	82.8±6.0	82.4±5.4	-0.3±1.8	0.37
WHR	0.96±0.02	0.96±0.02	-0.1±2.3	0.94
∑ 7 skinfolds (cm)	98.1±38.3	91.3±30.7	-4.5±13.2	0.13
Body density	1.07±0.01	1.07±0.01	0.2±0.5	0.13
Fat %	13.0±4.9	12.2±4.1	-4.2±12.7	0.11
Cardiovascular				
RHR (beats/min)	64.1±10.8	59.5±9.3	-4.0±18.2	0.055
SBP (mmHg)	124.0±12.1	124.8±7.8	1.1±10.5	0.77
DBP (mmHg)	72.0±6.7	69.5±7.0	-3.4±7.9	0.06
MAP (mmHg)	89.4±7.9	87.9±6.7	-1.3±8.8	0.42
PP (mmHg)	51.9±8.4	55.4±5.8	7.6±16.8	0.07
Total CHOL (mmol/L)	4.42±0.79	4.24±0.67	-3.3±9.3	0.06
Triglycerides (mmol/L)	0.97±0.35	0.95±0.48	-4.2±22.4	0.70
HDL-C (mmol/L)	1.34±0.24	1.31±0.20	-0.9±11.7	0.50
LDL-C (mmol/L)	2.64±0.78	2.48±0.61	-3.9±11.2	**0.047**
LDL-C/HDL-C (mmol/L)	2.06(1.5–2.8)	2.0(1.5–2.5)	-1.6±15.0	0.24

CHOL/HDL-C (mmol/L)	3.41(2.7–4.2)	3.31(2.7–4.2)	-2.0±9.1	0.35
Cardiorespiratory fitness				
VO_{2max} ($ml \cdot kg \cdot min^{-1}$)	49.5±4.7	51.6±3.9	4.7±10.1	**0.03**
Final treadmill Speed ($km \cdot hr^{-1}$)	19(18–21)	20(19–21)	4.0±5.1	**0.001**

Data are expressed as mean ± standard deviation or as mean (interquartile range) from paired t-tests or related samples Wilcoxon signed rank test, respectively.

Legend: ∑, sum of; % change, percentage change; BMI, Body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; CHOL, cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

889 Table 4. DNA methylation changes after four weeks of exercise training (≥10%, q≤0.005).

Location in relation to					***DNA methylation (%)***				
CpG	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before exercise**	**After exercise**	**Diff**	***P*-value**	***q*-value (≤0.005)**
cg19933985			S shelf	5	53.8±4.4	70.8±4.1	17	1.93×10^{-7}	1.49×10^{-5}
cg10633981	*C11orf58*	3'UTR	Open sea	11	49.5±6.7	66.4±5.1	16.9	2.13×10^{-8}	3.97×10^{-6}
cg02732134	*DNMBP*	5'UTR	Open sea	10	42.3±2.8	57.9±3.6	15.6	4.79×10^{-8}	6.26×10^{-6}
cg01287788	*SNORD94* *PTCD3*	TSS200 Body	Open sea	2	49.5±4.2	65.0±5.2	15.5	2.96×10^{-7}	1.96×10^{-5}
cg27379715			Open sea	2	55.1±3.3	70.0±3.3	14.8	8.39×10^{-9}	2.39×10^{-6}
cg17393016	*C17orf55*	TSS1500	N shore	17	54.2±13.2	68.7±15.9	14.5	0.002	0.005
cg02308712			Open sea	12	47.5±3.9	61.7±4.3	14.1	9.38×10^{-8}	9.45×10^{-6}
cg15897635			N shelf	1	30.7±6.6	44.4±5.3	13.8	0.0003	0.001
cg21937244	*CDC42BPB*	Body	Island	14	64.4±5.9	78.0±3.4	13.7	3.10×10^{-8}	4.86×10^{-6}

cg12578536			S Shelf	5	48.44.0	61.8±3.5	13.4	2.74×10^{-7}	1.86×10^{-5}
cg17931986	*COL11A2*	3'UTR	S shore	6	53.8±3.1	67.2±3.6	13.4	6.86×10^{-9}	2.16×10^{-6}
cg04205664	*CLEC2L*	Body	S shore	7	28.3±1.9	41.5±4.1	13.2	2.09×10^{-7}	1.57×10^{-5}
cg05239225	*AKAP13*	5'UTR	Open sea	15	46.6±7.3	59.5±4.3	12.9	0.0003	0.001
cg13810766	*PRKAG2*	Body	Open sea	7	33.6±1.9	45.9±3.1	12.3	2.72×10^{-9}	1.39×10^{-6}
cg12949141	*PCBD2*	Body	Open sea	5	35.1±5.6	47.1±4.9	12.1	8.56×10^{-5}	0.0007
cg02771392	*ZNF273*	TSS1500	Open sea	7	59.3±3.1	71.3±2.0	12	1.58×10^{-8}	3.35×10^{-6}
cg04250181	*SLCO1B3*	5'UTR	Open sea	12	65.0±6.0	76.9±3.5	11.9	1.90×10^{-6}	6.24×10^{-5}
cg01775802	*RGS6*	Body	Open sea	14	27.6±5.1	39.3±5.2	11.7	9.61×10^{-5}	0.0007
cg22870994			Open sea	11	44.3±2.3	55.9±2.4	11.6	1.06×10^{-7}	1.02×10^{-5}
cg14497545	*MAML3*	Body	Open sea	4	36.5±2.3	48.1±4.0	11.5	5.62×10^{-7}	2.95×10^{-5}
cg23817637	*CLRN3*	TSS1500	Open sea	10	60.3±6.2	71.8±2.6	11.5	0.0001	0.0009
cg18022036			Open sea	5	47.3±3.9	37.4±4.6	-10	0.0001	0.001
cg06611444			Open sea	4	70.1±5.5	60.2±5.4	-10	8.78×10^{-6}	0.0002

cg16859420			Open sea	8	31.6±5.4	21.6±3.9	-10	0.0002	0.001
cg20090957	*MAPKAPK5* *C12orf47*	TSS1500 Body	Island	12	34.1±3.1	24.1±2.4	-10	2.10×10^{-6}	6.63×10^{-5}
cg17502267	*BNIP2*	Body	Island	15	39.4±2.5	29.4±4.0	-10	2.59×10^{-5}	0.0003
cg22876894			*N shore	20	31.5±4.2	21.5±2.6	-10	1.25×10^{-7}	1.13×10^{-5}
cg18825597			N shelf	8	61.2±4.4	51.1±6.4	-10.1	0.001	0.004
cg16146432	*C3orf50*	TSS200	N shore	3	26.1±4.5	16.1±3.1	-10.1	1.52×10^{-6}	5.43×10^{-5}
cg17479131	*LOC401431*	Body	N shelf	7	54.2±2.3	44.1±4.4	-10.1	5.14×10^{-5}	0.0005
cg06180061	*C16orf91*	Body	S shelf	16	62.2±3.5	52.0±4.6	10.1	1.98×10^{-8}	3.84×10^{-6}
cg08339189	*GGTA1*	Body	N shore	9	21.8±1.7	11.6±2.3	10.2	1.71×10^{-6}	5.83×10^{-5}
cg17932934	*CALN1*	5'UTR 1st Exon	Open sea	7	72.9±2.1	62.7±5.7	10.2	3.92×10^{-5}	0.0004
cg14366742			Open sea	4	43.4±4.0	33.2±4.6	10.2	5.62×10^{-8}	6.85×10^{-6}
cg11381792	*TRAPPC10*	Body	Island	21	31.9±1.3	21.7±1.8	10.2	1.64×10^{-9}	1.12×10^{-6}
cg04669668	*BANP*	Body	Island	16	57.2±3.7	46.9±4.8	10.3	0.0001	0.0008
cg09700085	*SLC6A20*	5'UTR	Island	3	26.0±2.2	15.7±1.9	10.3	2.08×10^{-8}	3.93×10^{-6}

		1st Exon							
cg08206623	*CDKN1C*	TSS1500	Island	11	45.8±1.5	35.5±1.8	10.3	2.61×10^{-8}	4.38×10^{-6}
cg20144008			Island	2	45.3±2.1	35.0±2.4	10.4	1.11×10^{-8}	9.05×10^{-7}
cg07894334	*SOCS5*	TSS1500	†N shore	2	59.2±7.2	48.8±5.8	10.5	2.78×10^{-5}	0.0003
cg27130012			S shore	6	74.6±2.7	64.1±4.6	10.5	1.02×10^{-5}	0.0002
cg15386880			Island	16	50.5±4.8	40.0±7.5	10.5	0.0001	0.0009
cg00001793	*ETV6*	Body	Open sea	12	59.2±5.0	48.7±7.1	10.5	0.0001	0.0008
cg21265548	*NSDHL* *CETN2*	5'UTR TSS1500	‡S shore	X	46.3±8.1	35.7±7.1	10.6	0.001	0.004
cg05280527	*NRXN3*	3'UTR	S shore	14	47.7±3.0	37.1±3.5	10.6	9.17×10^{-8}	9.35×10^{-6}
cg02678768	*EVPL*	3'UTR	N shore	17	67.1±9.1	56.4±8.7	10.7	3.77×10^{-6}	9.51×10^{-5}
cg23917513	*LMX1A*	5'UTR Body	Open sea	1	66.1±4.1	55.4±5.5	10.7	3.92×10^{-5}	0.0004
cg13985765	*PCSK6*	Body	Open sea	15	68.7±6.4	57.9±6.4	10.8	0.0005	0.002
cg25152348	*NCAPH2*	5'UTR 1st Exon	Island	22	47.2±2.1	36.3±1.8	10.9	5.29×10^{-8}	6.62×10^{-6}

	LMF2	TSS1500							
cg16500605	*BAT3*	TSS1500	Island	6	31.9±3.5	20.9±1.4	11	1.05×10^{-6}	4.29×10^{-5}
cg01558909	*HBM*	TSS200	Island	16	14.8±9.0	3.8±0.5	11	0.0006	0.002
cg15694789	*STARD8*	5’UTR	S shore	X	38.3±4.6	27.3±3.3	11	2.69×10^{-6}	7.71×10^{-5}
cg05524038	*CSF1R*	TSS1500	Open sea	5	80.0±2.9	68.9±2.5	11.1	1.51×10^{-6}	5.42×10^{-5}
cg11123847	*ACTR10*	3’UTR	Open sea	14	80.9±3.1	69.8±4.3	11.2	1.14×10^{-5}	0.0002
cg14506366	*SLC6A3*	Body	Open sea	5	66.5±7.0	55.3±6.6	11.2	0.001	0.003
cg06082548	*NKX6-2*	Body	*Island	12	32.3±2.1	21.0±1.9	11.3	3.50×10^{-10}	5.28×10^{-7}
cg26979339	*RIC8B*	TSS1500	Island	12	35.7±1.5	24.3±2.2	11.4	1.45×10^{-8}	3.23×10^{-6}
cg25474648	*ZDHHC14*	3’UTR	S shore	6	74.2±2.5	62.7±4.8	11.5	2.62×10^{-6}	7.58×10^{-5}
cg01994308	*PLAG1* *CHCHD7*	5’UTR TSS1500	N shore	8	40.0±6.7	28.5±4.2	11.5	0.0005	0.002
cg12093976	*EGF*	TSS200	Open sea	4	34.3±2.5	22.8±3.0	11.6	7.18×10^{-9}	2.20×10^{-6}
cg12861974	*CXorf57*	TSS200	Island	X	33.4±4.8	21.8±2.9	11.6	3.04×10^{-6}	8.32×10^{-5}
cg20929922	*C5orf28*	TSS1500	S shore	5	37.7±3.1	26.0±3.4	11.7	3.23×10^{-5}	0.0004

cg19349861	*OPCML*	5'UTR 1st Exon	Open sea	11	34.7±3.0	22.9±2.3	11.7	1.62×10^{-8}	3.41×10^{-6}
cg03909902	*PRDM4*	5'UTR	Island	12	35.6±2.8	23.8±1.7	11.8	1.75×10^{-8}	3.54×10^{-6}
cg04981696	*ABCC1*	Body	S shore	16	41.5±4.3	29.5±4.0	12	3.30×10^{-6}	8.76×10^{-5}
cg05005382	*CHTF18*	Body	Island	16	68.5±3.3	56.4±5.0	12.1	0.0003	0.001
cg00556029	*MARCKSL1*	TSS200	Island	1	30.9±4.8	18.7±2.6	12.2	3.22×10^{-5}	0.0004
cg03252499			Open sea	11	63.3±7.4	51.0±5.3	12.3	0.001	0.004
cg25501666			N shelf	2	65.8±4.5	53.1±4.8	12.7	3.68×10^{-6}	9.35×10^{-5}
cg20982606	*UNG*	TSS1500 TSS200	Island	12	29.1±3.0	16.2±2.4	12.9	9.23×10^{-9}	2.52×10^{-6}
cg16379462	*SAMD4A*	Body	Open sea	14	64.6±3.8	51.6±6.4	12.9	0.0004	0.002
cg14266237	*KLF1*	3'UTR	N shore	19	49.8±6.9	36.8±8.3	13	3.93×10^{-8}	5.62×10^{-6}
cg21393587			N shore	X	74.4±2.4	61.4±4.0	13	5.53×10^{-8}	6.76×10^{-6}
cg06808467	*LOC339290* *C18orf18*	TSS1500 Body	Island	18	39.2±2.4	25.6±3.1	13.6	2.56×10^{-8}	4.36×10^{-6}
cg17092349	*DALRD3*	5'UTR	N shore	3	47.5±6.6	33.8±5.8	13.7	5.10×10^{-5}	0.0005

	NDUFAF3	TSS1500							
	MIR425	TSS1500							
	MIR191	TSS200							
cg26081875			Open sea	6	36.2±7.9	22.3±5.3	13.9	0.001	0.004
cg01525244	*CBX7*	TSS200	N shore	22	30.3±1.2	16.3±1.9	14	4.20×10^{-10}	5.96×10^{-7}
cg09844907	*MPV17L*	5'UTR 1st Exon	Island	16	34.0±1.5	19.4±2.6	14.6	1.87×10^{-8}	3.70×10^{-6}
cg27494111			N shore	19	67.6±5.1	52.5±10.4	15.1	3.25×10^{-6}	8.68×10^{-5}
cg26186549			S shore	6	68.7±11.4	53.3±11.9	15.4	2.77×10^{-5}	0.0003
cg16524049	*LMX1A*	Body	Island	1	43.1±2.7	27.1±4.6	16	4.54×10^{-6}	0.0001

Data are expressed are beta values ± standard deviation.

Legend: Chr, chromosome number; Diff, difference; 3', three prime; 5', five prime; UTR, untranslated region; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site; *DMR, differentially methylated region; †CDMR, cancer differentially methylated region; ‡RDMR, reprogrammed differentially methylated region.

894 Table 5. DNA methylation changes after four weeks of exercise training (≥20%, q≤0.005).

	Location in relation to				***DNA methylation (%)***				
CpG	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before exercise**	**After exercise**	**Diff**	***P*-value**	***q*-value (≤0.005)**
cg21036194	*SNCAIP*	Body	Open sea	5	24.1±23.1	86.2±1.8	62.1	5.05×10^{-7}	2.76×10^{-5}
cg22588144	*ISM1*	TSS1500	Island	20	4.9±1.0	53.1±8.3	48.2	1.69×10^{-12}	5.35×10^{-8}
cg01309395	*HLA-DPB2*	Body	Open sea	6	37.9±34.7	82.1±4.1	44.2	0.0004	0.002
cg07658590	*SLC19A1*	TSS1500	S shore	21	15.6±18.2	47.6±3.9	32.0	0.0004	0.002
cg18531559	*ULK4*	TSS200	S shore	3	8.4±1.9	36.1±0.6	27.7	1.70×10^{-11}	1.56×10^{-7}
cg11950805	*CDC20*	TSS1500	Island	1	1.7±0.5	25.8±15.0	24.1	5.47×10^{-5}	0.0005
cg26919805	*PPPDE2*	5'UTR 1st Exon	Island	22	1.9±0.5	25.2±18.2	23.3	0.001	0.003
cg09459740	*XRCC6*	TSS1500	Open sea	11	74.1±1.2	96.2±5.2	22.1	9.12×10^{-8}	9.34×10^{-6}
cg07170824	*ACVRL1*	5'UTR 1st Exon	Island	12	52.7±3.1	32.7±5.3	-20.0	2.51×10^{-7}	1.75×10^{-5}
cg16700025	*CMIP*	Body	Open sea	16	62.2±15.4	36.1±0.6	-26.0	0.0001	0.001
cg18684755	*RYR1*	Body	S shelf	19	86.9±1.6	56.7±25.4	-30.2	0.002	0.005
cg06390613	*TMEM198*	3'UTR	N shelf	2	76.2±2.0	43.9±18.	-32.3	0.0001	0.0009

						2			
cg03229033			Island	1	44.5±5.8	12.0±21.6	-32.4	0.0001	0.0009
cg15224432	*IGFL3*	TSS1500	Open sea	19	73.8±1.8	36.1±0.6	-37.7	7.17×10^{-16}	4.55×10^{-11}
cg04600795			Open sea	6	88.7±1.2	45.1±20.9	-43.7	1.50×10^{-5}	0.0002
cg10580110	*KRT6A*	TSS1500	Open sea	12	88.5±1.7	44.8±29.6	-43.7	1.40×10^{-5}	0.0002
cg08975528	*ZBTB12*	3'UTR	Island	6	74.6±2.9	19.8±34.1	-54.7	0.0002	0.001
cg17415355	*TOLLIP*	Body	S shelf	11	86.5±1.5	24.9±35.8	-61.6	8.60×10^{-5}	0.0007
cg24810735	*ANGPT1*	Body	Open sea	8	79.3±3.5	16.5±32.2	-62.8	1.95×10^{-5}	0.0003

Data are average beta-values ± standard deviation.

Legend: Chr, chromosome number; Diff, difference; 3', three prime; 5', five prime; UTR, untranslated region; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site.

908 Table 6. Pathways modulated by exercise training ($q \leq 0.05$).

Pathway name	Enrichment score	Enrichment *P*-value	Enrichment *q*-value	# of Genes	Pathway ID
Endocytosis	16.44	7.23×10^{-8}	1.22×10^{-5}	115	kegg pathway 211
HTLV-I infection	13.52	1.35×10^{-6}	0.0001	154	kegg pathway 251
Wnt signaling pathway	11.93	6.61×10^{-6}	0.0003	88	kegg pathway 142
Proteoglycans in cancer	11.71	8.21×10^{-6}	0.0003	122	kegg pathway 164
Pathways in cancer	11.15	1.44×10^{-5}	0.0005	171	kegg pathway 128
Axon guidance	10.8	2.04×10^{-5}	0.0006	77	kegg pathway 176
Gap junction	10.44	2.92×10^{-5}	0.0007	58	kegg pathway 127
Regulation of actin cytoskeleton	9.97	4.66×10^{-5}	0.001	120	kegg pathway 179
Glutamatergic synapse	9.05	0.0001	0.002	67	kegg pathway 182
Calcium signaling pathway	9.03	0.0001	0.001	101	kegg pathway 263
Hippo signaling pathway	9.02	0.0001	0.002	92	kegg pathway 20
MAPK signaling pathway	8.93	0.0001	0.002	129	kegg pathway 119
p53 signaling pathway	8.6	0.0002	0.002	32	kegg pathway 149
Melanogenesis	8.28	0.00025	0.003	64	kegg pathway 206
Cholinergic synapse	7.94	0.00035	0.004	69	kegg pathway 237

Amphetamine addiction	7.53	0.0005	0.006	37	kegg pathway 50
Focal adhesion	7.13	0.0008	0.008	103	kegg pathway 5
Circadian entrainment	7.07	0.0008	0.008	56	kegg pathway 51
Basal cell carcinoma	7.02	0.0009	0.008	39	kegg pathway 35
Dopaminergic synapse	6.99	0.0009	0.008	68	kegg pathway 91
Dilated cardiomyopathy	6.34	0.002	0.01	53	kegg pathway 134
Epstein-Barr virus infection	6.05	0.002	0.02	113	kegg pathway 252
Cocaine addiction	5.92	0.003	0.02	28	kegg pathway 107
Long-term potentiation	5.88	0.003	0.02	35	kegg pathway 208
Hedgehog signaling pathway	5.32	0.005	0.03	33	kegg pathway 167
Chronic myeloid leukemia	5.17	0.006	0.03	42	kegg pathway 240
Melanoma	5.17	0.006	0.03	42	kegg pathway 249
Fc gamma R-mediated phagocytosis	5.16	0.006	0.03	45	kegg pathway 77
Glioma	4.95	0.007	0.04	38	kegg pathway 161
B cell receptor signaling pathway	4.95	0.007	0.04	38	kegg pathway 188

ECM-receptor interaction	4.94	0.007	0.04	47	kegg pathway 41
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	4.92	0.007	0.04	17	kegg pathway 248
Other types of O-glycan biosynthesis	4.92	0.007	0.04	17	kegg pathway 38
PI3K-Akt signaling pathway	4.74	0.009	0.04	166	kegg pathway 45
Oocyte meiosis	4.69	0.009	0.04	60	kegg pathway 22
T cell receptor signaling pathway	4.6	0.01	0.046	54	kegg pathway 70
Aminoacyl-tRNA biosynthesis	4.59	0.01	0.046	27	kegg pathway 116
Spliceosome	4.5	0.01	0.049	75	kegg pathway 131
Small cell lung cancer	4.48	0.01	0.049	42	kegg pathway 114
Progesterone-mediated oocyte maturation	4.36	0.01	0.05	50	kegg pathway 254

Legend: #, number.

914 Table 7. *MIR21* and *MIR210* DNA methylation after four weeks of exercise training (q<0.05).

Location in relation to					***DNA methylation (%)***				
CpG	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before Exercise**	**After exercise**	**Diff**	***P*-value**	***q*-value (≤0.005)**
cg27023597	*MIR21*	TS1500	Open Sea	17	67.4±3.6	64.4±4.0	-3	0.007	0.01
cg04276626	*MIR21*	TSS200	Open Sea	17	79.3±3.4	76	-3.3	0.008	0.01
cg02515217	*MIR21*	TSS200	Open Sea	17	80.0±2.4	78.5±2.1	-1.5	0.009	0.01
cg07181702	*MIR21*	Body	Open Sea	17	78.4±3.0	74.6±3.9	-3.8	0.02	0.03
cg02471760	*MIR210*	TSS1500	S Shore	11	54.6±4.7	50.4±4.6	-4.2	0.002	0.004
cg08200293	*MIR210*	TSS1500	Island	11	13.2±1.2	11.8±2.1	-1.4	0.01	0.02
cg05858042	*MIR210*	TSS1500	Island	11	24.5±3.9	21.7±5.1	-2.7	0.005	0.009

cg07410811	*MIR210*	TSS1500	Island	11	8.1±1.2	6.7±1.5	-1.4	0.003	0.007
cg15482500	*MIR210*	TSS200	Island	11	11.6±0.7	9.3±0.7	-2.3	9.39×10^{-5}	0.0007
cg01277369	*MIR210*	TSS200	Island	11	7.5±1.1	6.2±0.9	-1.3	0.007	0.01
cg03880841	*MIR210*	Body	Island	11	18.5±0.01	15.9±1.2	-2.6	6.2×10^{-5}	0.0005

Data are average beta-values ± standard deviation.

Legend: Chr, chromosome number; Diff, difference; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site.

Figures

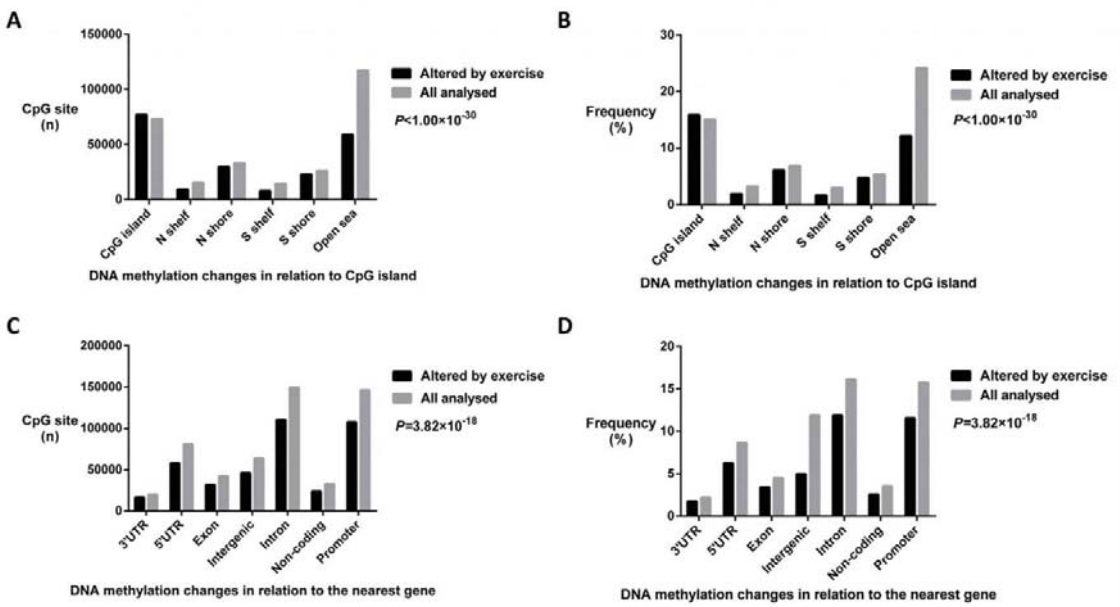

Figure 1. DNA methylation changes in relation to CpG islands (A and B) and the nearest gene (C and D), respectively.

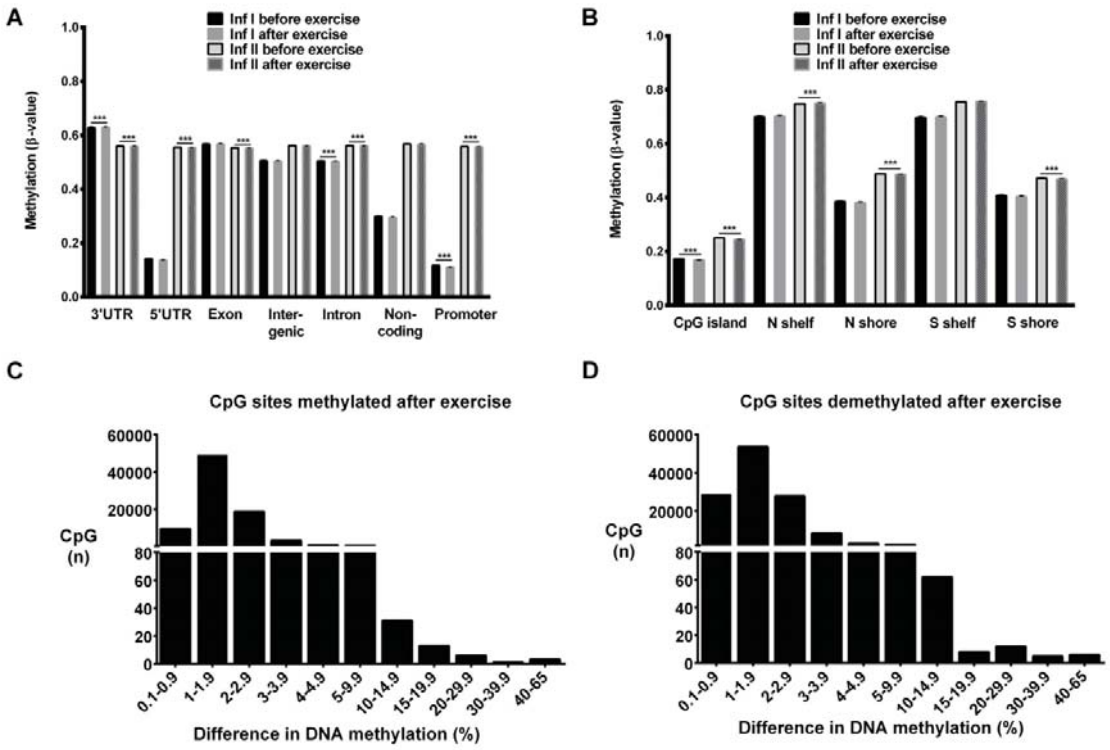

Figure 2. Whole-genome DNA methylation changes caused by exercise.

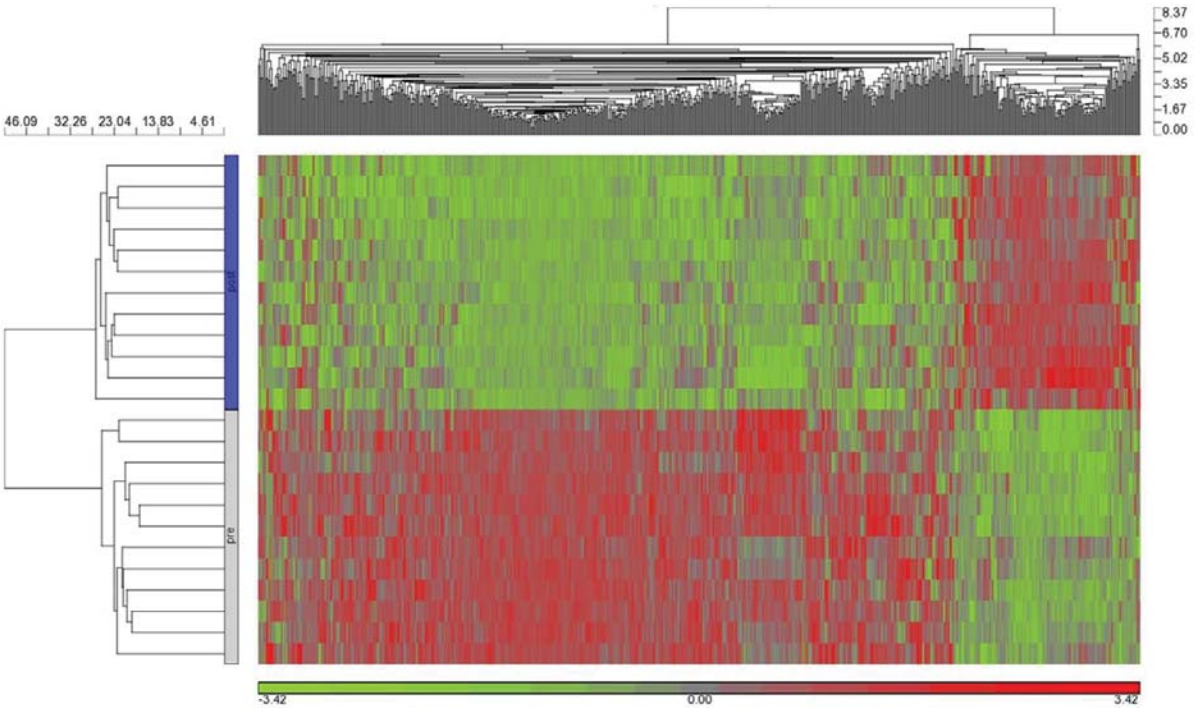

Figure 3. Hierarchical clustering of CpG methylation before and after exercise training.

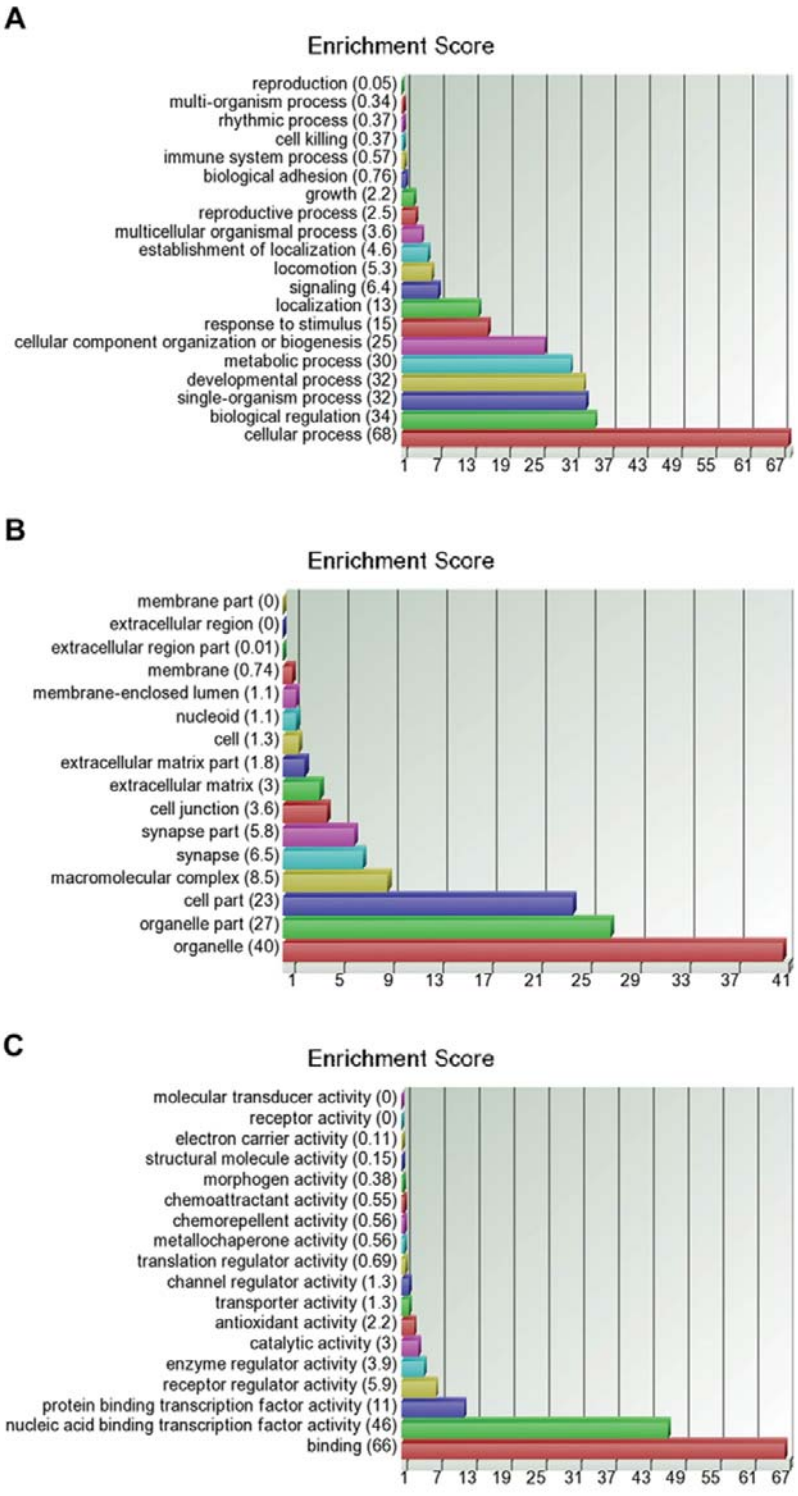

Figure 4. Gene ontology for genes with CpG DNA methylation changes after exercise.

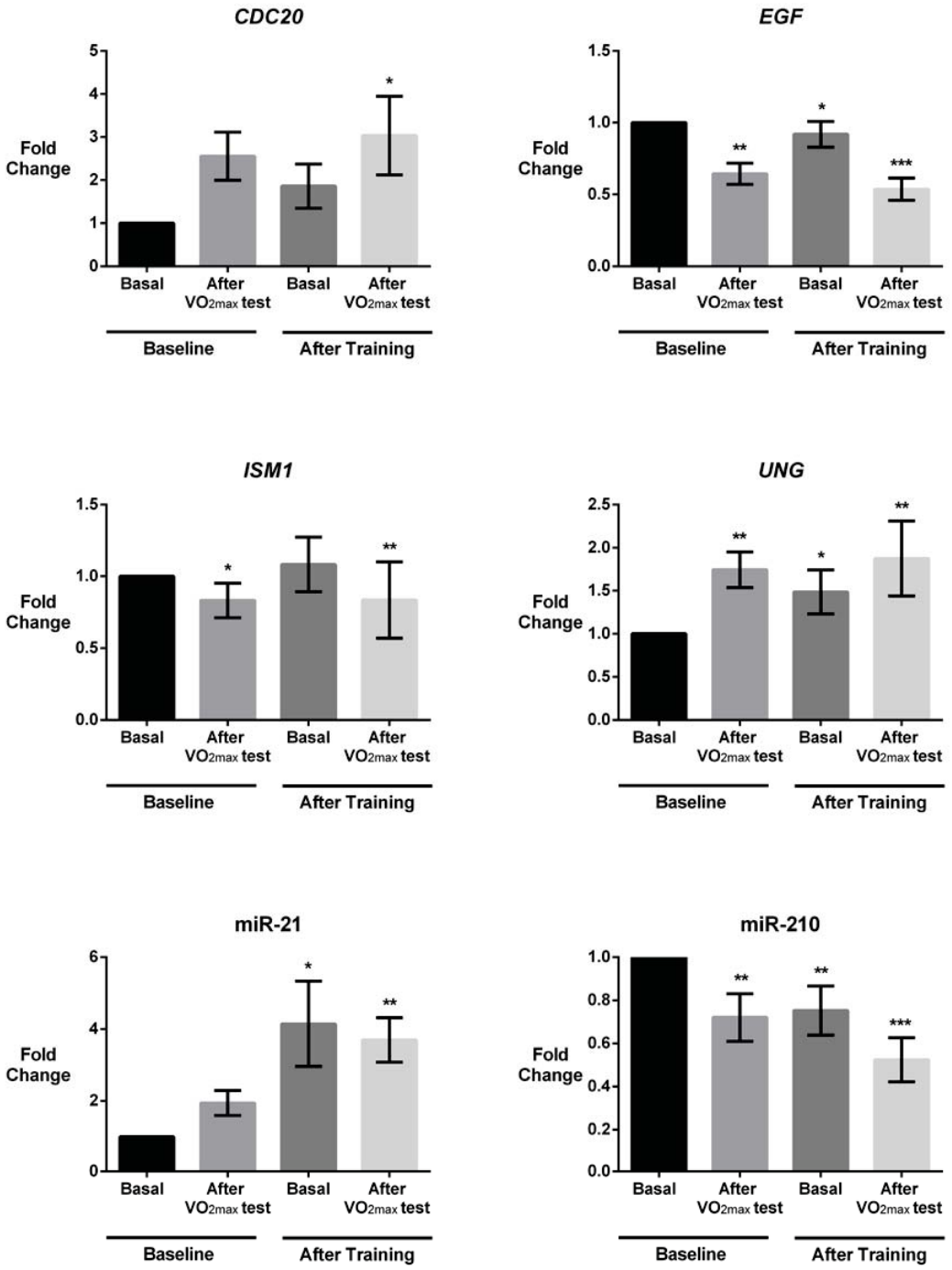

Figure 5. Gene and miRNA expression changes caused by exercise.

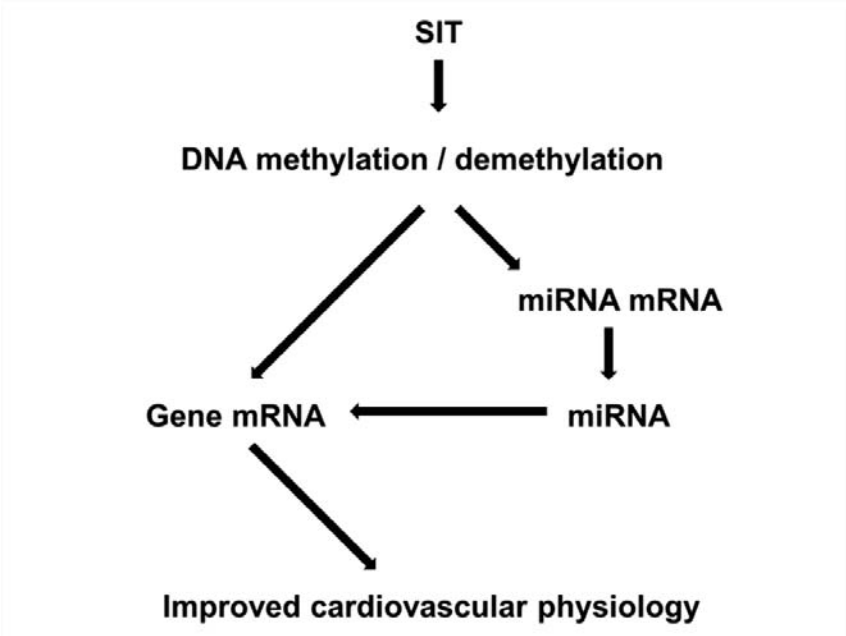

Figure 6. Schematic of DNA methylation regulation on cardiovascular physiology.