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Effects of age and sex on epigenetic modification induced by an acute physical exercise

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Abstract

It has been observed that, after 2 hours of aerobic exercise, plasma interleukin-6 (IL-6) increases whereas nuclear concentrations of enzyme DNA methyltransferase (DNMT) 3B significantly decreased in peripheral blood mononuclear cells (PBMCs), with no change observed in DNMT3A. The aim of the present study was to detect differences in these changes induced by exercise in plasma IL-6 levels as well as in PBMC nuclear concentrations of DNMT3A and DNMT3B, in relation to age and sex. Four groups were studied: 12 young men (24.8 ± 1.77 years old), 12 young women (23.8 ± 1.81 years old), 12 adult men (45.8 ± 1.82 years old), 12 adult women (mean 44.5 ± 2.07 years old). Participants had to run at 60% of maximal oxygen consumption (VO_{2max}) for 120 minutes, interspersed with sprints at 90% of VO_{2max} for the last 30 seconds of every 10 minutes. About 250 μL of PBMCs (1×10^6 cells) were treated with 100 μL of either pre-exercise plasma or post-exercise plasma and nuclear DNMT3A and DNMT3B concentrations were quantified. No change in nuclear concentration of DNMT3A following the exercise was observed. Conversely, nuclear concentrations of DNMT3B significantly decreased, with a reduction of about 78% in young men, 72% in young women, 61% in adult men, and 53% in adult women. Moreover, a strong positive correlation between the nuclear concentration of DNMT3B in PBMC following stimulation with post-exercise plasma and post-exercise plasma concentrations of IL-6 was observed in all the 4 studied groups. This study confirms that a single bout of endurance exercise is sufficient to decrease nuclear concentrations of DNMT3B and thus protein upregulation. Moreover, the epigenetic mechanisms induced by exercise apparently cause more intense changes in men than in women and that, in both of them, this effect seems to decrease with age.

Abbreviations: CpG = cytosine-phosphate-guanine, DNMT = DNA methyltransferase, ERβ = estrogen receptor beta, IL-6 = interleukin-6, PBMC = peripheral blood mononuclear cell, SD = standard deviation, TDG = thymine DNA glycosylase, VO_{2max} = maximal oxygen consumption.

Keywords: acute exercise, age, DNA methyltransferase, epigenetic regulation, sex, interleukin-6

1. Introduction

The term epigenetics defines variations of phenotypic traits that influence how cells express genes without changing the DNA sequence.[1] Methylation of DNA is the epigenetic modification most amply investigated, and is characterized by the addition of a methyl group to the nucleotide cytosine, creating 5-methylcytosine, with the intervention of the enzyme DNA methyltransferase (DNMT).[2] This process occurs predominantly at cytosine-phosphate-guanine (CpG) dinucleotides; CpG islands are strongly represented in gene promoter regions[3] where are characterized for their low level or complete absence of methylation.[4]

Hypermethylation at these CpG-rich gene promoter regions is often linked with transcriptional silencing, whereas hypomethylation is usually linked with active gene transcription.[5] The methylation process is regulated by several DNMTs; in particular, DNMT1 adds a methyl group at hemimethylated DNA, while DNMT3A and DNMT3B methylates preferentially unmethylated CpG dinucleotides.[6]

A number of studies showed that enduring exercise is capable to modify the DNA methylation in several tissues[7–10] whereas, concerning the effect of an acute exercise on DNA methylation, Barrès et al[11] described that a single maximal exercise was capable to induce in humans hypomethylation of vastus lateralis muscular cells.

Connolly et al[12] observed that a single bout of heavy exercise substantially alters in peripheral blood mononuclear cells (PBMCs) gene expression, characterized in many cases by a brisk activation and deactivation of genes associated with stress, inflammation, and tissue repair.

It has been recently observed that, after 120 minutes of treadmill running at an intensity of 60% of individual velocity at maximal oxygen consumption (vVO_{2max}) interspersed with

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Table 1

Baseline characteristics of the volunteers (mean ± standard deviation).

	Young men n = 12	Young women n = 12	Adult men n = 12	Adult women n = 12
Age, y	24.8 ± 1.77	23.8 ± 1.82	45.8 ± 1.82	44.5 ± 2.07
Height, cm	173.4 ± 3.90	168.3 ± 4.25	171.0 ± 4.29	166.2 ± 3.64
Weight, kg	72.4 ± 3.18	57.8 ± 4.13	70.9 ± 4.56	56.2 ± 4.22
Body mass index, $kg\,m^{-2}$	24.1 ± 0.46	20.4 ± 0.66	24.2 ± 0.89	20.3 ± 0.74
VO_{2max}, $mL\,kg^{-1}\,min^{-1}$	50.1 ± 3.62	39.2 ± 2.44	44.3 ± 2.62	33.6 ± 2.78

Participants' characteristics (mean ± standard deviation). VO_{2max} = maximal oxygen consumption.

30-second sprints at 90% of vVO_{2max} every 10 minutes, nuclear concentrations of DNMT3B significantly decreased in PBMCs, with no change observed in DNMT3A.[13] Various concentrations of Interleukin-6 (IL-6) recombinant protein caused an elevation in both DNMT3A and DNMT3B nuclear concentration compared with the blank control. The contradictory results between exercise and stimulation with IL-6 recombinant protein suggested to the authors that other circulating substances may also influence the nuclear concentrations of these enzymes.

It has been observed that the efficiency of epigenetic mechanisms is affected by aging[14] and there is evidence that estrogen receptor beta (ERβ) plays a role in regulating DNA methylation at specific genomic loci.[15] The authors proposed that ERβ binds to regulatory regions of target genes and recruits 10 to 11 translocator proteins and thymine DNA glycosylase (TDG) to these places. This interaction enhances gene expression on one hand and prevents DNA methylation on the other hand; these actions of estrogens suggest a possible sex differences in epigenetic adaptations.

The aim of the present study, therefore, was to detect potential differences in the exercise-induced changes in PBMC nuclear concentrations of the de novo DNA methyltransferases DNMT3A and DNMT3B in relation to age and sex.

2. Materials and methods

2.1. Participants

Forty-eight individuals of both sexes, regularly practicing recreational sport activity, volunteered to join in the exercise trial. The anthropometric characteristics of the participants are presented (mean values ± standard deviation [SD]) in Table 1. Participants gave written informed consent, and all the experiments were approved by the Ethical Committee of the University of Milan (number 15/16).

Participants were divided into 4 groups of 12 individuals each. The first group was formed by men with an age between 20 and 30 years (young men), the second group formed by women with an age between 20 and 30 years (young women), the third group formed by men with an age between 40 and 50 years (adult men), and the fourth group formed by women with an age between 40 and 50 years (adult women). All participants were required to be in possession of a certificate of suitability to competitive sports issued by a specialist in Sports Medicine. Young as well as adult women were not in menopause, did not use oral contraceptive and were studied in midluteal phase of their menstrual cycle.

2.2. Experimental protocol

The following protocol has previously been reported elsewhere[13] and has been shown to elicit a significant transient increase in plasma IL-6.

2.3. VO_{2max} assessment

VO_{2max} was defined as the highest value for VO_2 during the exercise. Achievement of VO_{2max} was confirmed by the following criteria: >90% of their age predicted maximal heart rate; and plateau of VO_2.[16] An open circuit spirometer Fitmade MED (Cosmed s.r.l. Italy) was used to collect and assess metabolic parameters. During the test, carried out by using a motorized treadmill (Excite Run 1000, Technogym, Italy), heart rate was measured by using a Polar (Gays Mills, WI) heart rate monitor.

2.4. Exercise protocol

The main experiment was carried out after a rest period of 7 days after the VO_{2max} assessment. The experimental conditions were explained to all volunteers; in particular, each participant had to avoid exercise, alcohol, and caffeine for 24 hours before the test; spend a pretesting overnight 12-hour fast, during which he/she was authorized to drink water ad libitum. The day of experiment, participants arrived in the lab at 09:00; height and weight were measured and 22.5 mL of blood was collected into ethylenediaminetetraacetic acid containing vacutainers (Becton Dickinson, Oxford, UK) from median cubital vein. Each participant had to run at 60% of vVO_{2max} for 120 minutes, interspersed with sprints at 90% of vVO_{2max} for the last 30 seconds of every 10 minutes. At the conclusion of the run, a further blood sample was immediately taken as previously described. The blood samples collected before and after the exercise were centrifuged at $1700 \times g$ for 10 minutes at 4 °C to separate the plasma and stored at −80 °C.

2.5. Interleukin-6 assay

Plasma concentrations of IL-6 before and after the exercise were quantified by using a QuantiGlo Human IL-6 ELISA (R&D Systems, Minneapolis, MN), following the standard manufacturer's guidelines. All samples were analyzed in duplicate, with assay sensitivity of 0.4 pg/mL, and assay detection range between 0.50 and 1500 pg/mL. Intra-assay coefficient of variation was calculated as 4.5%.

2.6. Peripheral blood mononuclear cells viability

PBMC viability was measured following incubation with exercise-conditioned plasma. As PBMCs source, whole blood from resting participants in all experimental groups was kept into 6 10 mL lithium heparin vacutainer tubes (Becton Dickinson, Oxford, UK). PBMCs were isolated by centrifugation of a LeucoSep centrifuge tube (Greiner Bio One, Frickenhausen, Germany) containing Lymphoprep (Stemcell Technologies, Vancouver, Canada). Cells were counted in a haemocytometer and opportunely diluted at the final density of 4×10^6 cells/mL. About 1×10^6 PBMCs (250 μL) were incubated at 37 °C for 4

hours with 100 μL of either pre-exercise plasma or post-exercise plasma, added to 150 μL of RPMI-1640 medium and penicillin/streptomycin/glutamine solution (Sigma–Aldrich, Milan, Italy). The whole process was repeated 3 times. Both before and after the incubation, PBMCs were mixed with trypan blue solution (Sigma–Aldrich, Milan, Italy) in a 1:1 ratio. The number of live and dead cells was then counted using a hemocytometer under an inverted microscope (Leica).

2.7. Nuclear protein extraction and quantification

PBMCs were treated as described above. Specifically, 1×10^6 PBMCs (250 μL) were incubated at 37°C for 4 hours with a mixture of 100 μL of either pre-exercise plasma or post-exercise plasma, and 150 μL of RPMI-1640 medium and penicillin/streptomycin/glutamine solution (Sigma–Aldrich, Milan, Italy). Nuclear proteins were extracted by using the Episeeker Nuclear Extraction Kit (Abcam, Cambridge, UK) and following the manufacturer's guidelines.

Protein quantification was estimated using the Bradford assay and reported as amount of protein per μL of nuclear extract (Quick Start Bradford Protein Assay, Bio Rad, CA). Samples, blank and scalar dilutions of a reference protein were read at an absorbance of 595 nm in a microplate reader (Synergy HT, Bio Tek, VT). To plot the data, the average blank value was subtracted from the sample absorbance, and a standard curve was generated.

2.8. DNMT quantification

Concentrations of nuclear DNMT3A and DNMT3B were quantified following the manufacturer's guidelines (Epiquik DNMT3A/B Assay Kit, Epigentek, New York, NY). A standard curve was plotted by using different dilutions of the respective DNMT standards. All assays were repeated in duplicate. Plates were read at a wavelength of 450 nm with a reference wavelength of 655 nm. Intra assay coefficient of variation was calculated as 3.9% for DNMT3A and 9.9% for DNMT3B

2.9. Statistical analysis

Data were collected and averaged, and then compared by using two-way analysis of variance followed by Tukey multiple comparisons test. Moreover, the linear regression and the correlation coefficient of Pearson were also calculated. Significance was set at $P < .05$. All descriptive statistics are reported as mean ± standard deviation (SD). All analyses were performed by using GraphPad Prism version 6.03 for Windows (GraphPad Software, San Diego, CA).

3. Results

The participants were individuals regularly practicing recreational sport activity showing a relatively high level of aerobic fitness, reflected by a mean value of their VO_{2max} (expressed as $ml\,kg^{-1}\,min^{-1}$) of approximately 50.1 (±3.62) in young men, 39.2 (±2.44) in young women, 44.3 (±3.62) in adult men, and 33.6 (±2.78) in adult women.

Figure 1 shows that in each condition, there was no statistically significant difference in cell viability before and after incubation for 4 hours at 37°C.

The effects of cell stimulation with plasma isolated following 120 minutes of treadmill running, on subsequent changes in nuclear concentrations of the de novo DNA methyltransferases DNMT3A and DNMT3B are illustrated visually in Fig. 2. The same figure also shows plasma concentrations of IL-6 before and after the exercise.

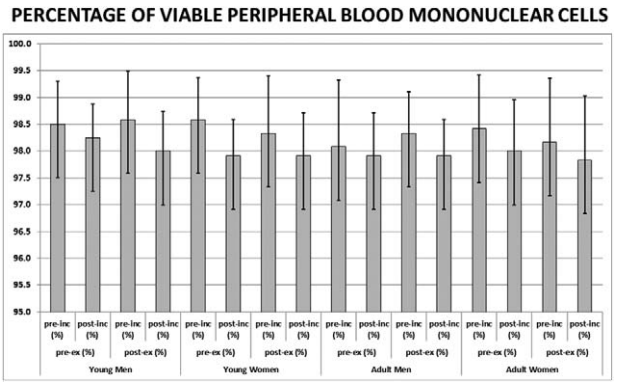

Figure 1. Percentage (mean values ± standard deviation, SD) of viable peripheral blood mononuclear cells before and after incubation period. Pre-inc = before the incubation, post-inc = after the incubation, pre-ex = before the exercise, post-ex = after the exercise.

Baseline concentrations of DNMT3B were significantly higher than DNMT3A ($P = .02$), showing that DNMT3B is more immediately abundant within PBMC nuclei.

No change was observed in nuclear concentration of DNMT3A following the exercise stimulus in any of the 4 groups studied.

Conversely, however, nuclear concentrations of DNMT3B significantly decreased in all the 4 studied groups, with a reduction immediately following the exercise bout of about 78% in young men, 72% in young women, 61% in adult men, and 53% in adult women. Statistical analysis (unpaired *t* test) showed that the minor decrease observed in Young Women than in Young Men is highly significant ($P < .0001$), and it is also significant the smaller decrease detected in adult women than adult men ($P = .0005$). Moreover, young men exhibited significantly more pronounced reductions than adult men ($P < .0001$) and young women exhibited more most marked decreases compared with adult women ($P < .0001$).

At the same time, plasma concentration of IL-6 significantly increased in all the 4 groups studied, with an increase immediately following the exercise bout of about 35 times in young men, 27 times in young women, 25 times in adult men, and 12 times in adult women. Statistical analysis (unpaired *t* test) showed that the minor increase observed in young women than in young men is significant ($P = .0324$), and it is also significant the smaller increase detected in adult women than adult men ($P < .0001$). Moreover, young men showed significantly more pronounced increases than adult men ($P = .0311$), and young women exhibited more most marked increases compared with adult women ($P < .0001$).

At this point, possible correlations between exercise-induced increase in plasma IL-6 and reductions of nuclear concentrations of DNMT3B were analyzed as well as possible correlation between these 2 exercised-induced changes and VO_{2max} of the studied participants.

As can be seen in Fig. 3A, a strong positive correlation between the nuclear concentration of DNMT3B in PBMC following stimulation with post-exercise plasma and post-exercise plasma concentrations of IL-6 was observed in all the 4 studied groups. In

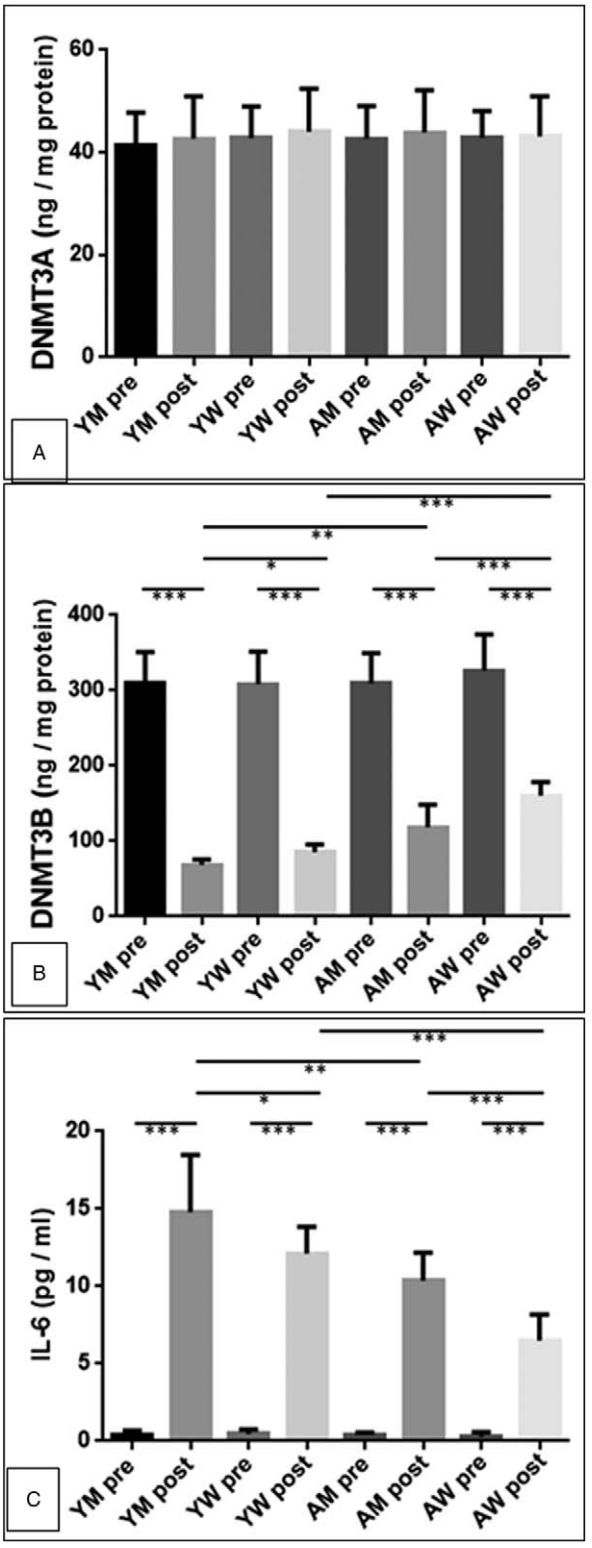

Figure 2. A: Mean values (±SD) of nuclear concentration of DNA methyltransferase 3A (DNMT3A) in peripheral blood mononuclear cell (PBMC) following stimulation with pre and post-exercise plasma. B: Mean values (±SD) of nuclear concentration of DNA methyltransferase 3B (DNMT3B) in PBMC following stimulation with pre and post-exercise plasma. C: Mean values (±SD) of pre- and post-exercise plasma concentrations of interleukin-6 (IL-6). AM = adult men, AW = adult women, YM = young men, YW = young women. Symbols representing statistical significance of the differences (two-way ANOVA): $^{*}P > .05$; $^{**}P > .01$; $^{***}P > .001$. ANOVA = analysis of variance.

fact, for young men linear regression displays a R square value of 0.7974 ($P < .0001$), for young woman a R square value of 0.7135 ($P = .0005$), for adult men a R square value of 0.5275 ($P = .0075$) and, finally, for adult women a R square value of .6098 ($P = .0027$).

Conversely, as showed by Fig. 3B and C, none significant correlation was observed between post-exercise plasma concentrations of IL-6 and VO_{2max} as well as between VO_{2max} and nuclear concentration of DNMT3B in PBMC following stimulation with post-exercise plasma, respectively.

4. Discussion

The present study was carried out to investigate changes in the concentrations of the de novo DNA methyltransferases DNMT3A and DNMT3B following stimulation of PBMCs with plasma isolated before and after an acute bout of intense treadmill exercise in 4 groups of participants, regularly practicing recreational sport activity, having different age and sex. We observed that while DNMT3A concentrations remained unaltered, a significant reduction in the nuclear concentration of DNMT3B was observed in all 4 groups, which is consistent with previous findings.[13] These data confirm that the 2 de novo methyltransferases have distinct roles in response to exercise, and highlights a possible mechanism by which exercise may be able to acutely alter levels of DNA methylation.

Robson-Ansley et al[17] reported that plasma interleukin-6 (IL-6) may be associated with DNA methylation. In particular, they analyzed the effect on DNA methylation of PBMCs of a 120-minute treadmill run at 60% of maximal oxygen consumption (VO_{2max}), intermingled every 10 minutes with sprints at 90% of VO_{2max} for the last 30 seconds (capable to cause increases in plasma IL-6[18]), followed by a 5-km time trial,. It was observed that increases in plasma IL-6 concentration induced by the exercise were significantly associated with the methylation levels of 11 different genes.

Probably, IL-6 alters DNA methylation by changing DNMT expression and cellular translocation. Hodge et al[19] reported that increased DNMT1 activity may be produced by protein kinase B (AKT)-dependent phosphorylation of the DNMT1 nuclear localization sequence, so consenting nuclear translocation, after incubation of human colorectal carcinoma cells with IL-6. However, there is at yet no experimental evidence that IL-6 directly interacts with DNMTs in PBMCs.

However, the absence in the present investigation of a direct measure of genomic or gene-specific methylation does not allow to know whether the decrease of DNMT3B cause directly the change of DNA methylation.

The above described effects, induced by an acute exercise, were found to have different intensities depending on age and sex. In fact, the reduction of nuclear concentrations of DNMT3B immediately following the exercise bout was of about 78% in young men, 72% in young women, 61% in adult men, and 53% in adult women. Similarly, increase of plasma concentration of IL-6 immediately following the exercise bout was of about 35 times in young men, 27 times in young women, 25 times in adult men, and 12 times in adult women.

Moreover, in the present study a strong positive correlation between the nuclear concentration of DNMT3B in PBMC following stimulation with post-exercise plasma and the post-exercise plasma concentrations of IL-6 was observed in all the 4 studied groups. Conversely, none significant correlation was observed between post-exercise plasma concentrations of IL-6

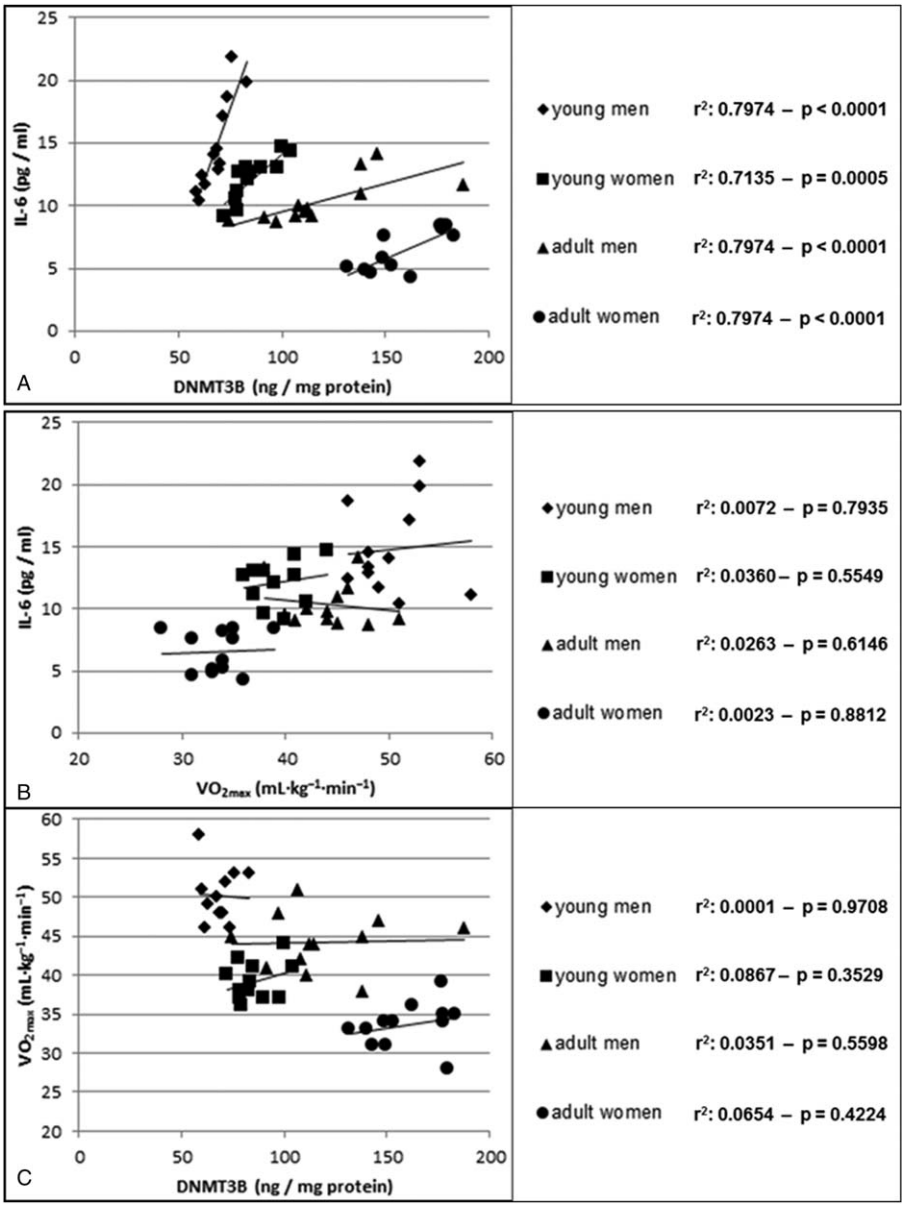

Figure 3. A: Linear regression between nuclear concentration of DNMT3B in PBMC following stimulation with post-exercise plasma and post-exercise plasma concentrations of IL-6. B, Linear regression between maximal aerobic power (VO_{2max}) of the participants with post-exercise plasma concentrations of IL-6. C, Linear regression between nuclear concentration of DNMT3B in PBMC following stimulation with VO_{2max} of the participants. The r^2 value from the Pearson correlation coefficient and the *P* value are shown on the right. IL-6 = interleukin-6, pre-inc = before the incubation, post-inc = after the incubation, pre-ex = before the exercise, post-ex = after the exercise.

and VO_{2max} as well as between VO_{2max} and nuclear concentration of DNMT3B in PBMC following stimulation with post-exercise plasma.

This study confirms that circulatory factors found in the plasma following a single bout of endurance exercise are sufficient to decrease nuclear concentrations of DNMT3B, which points to IL-6 as a contributor to exercise-induced gene promoter hypomethylation, and thus protein upregulation, as previously suggested.[13] Moreover, the present results allow to conclude that the epigenetic mechanisms induced by exercise tends to decrease with age and cause more intense changes in men than in women.

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