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Acute effects of endurance exercise on Global DNA methylation after a high intensity and moderate intensity intervention – A methodological study

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Abstract

Background: DNA methylation is currently the most examined epigenetic modification and is involved in the development of various diseases. Epigenetic processes can be positively influenced by lifestyle factors such as sports. The current literature base presents studies with different approaches in terms of exercise, intensity, duration, analyzed tissue, and analyzing methods of exercises on global DNA methylation. This methodological study aims to investigate the effectiveness of physical endurance training on global DNA methylation in leukocytes at two different intensities and to detect whether the Methyl Flash™ Global DNA methylation ELISA Kit [#P-1030, Epigenetic©] is able to measure small alterations in methylated DNA.

Methods: Twelve healthy subjects (age: 23.75 ± 2.56 years) completed two interventions of different intensities on a bicycle ergometer. After determining an individual peak power. Participants completed interventions with high intensity (85% peak power) and moderate-intensity (50 % peak power) at an interval of one week. During these two training days, peripheral venous blood was taken from the subjects one hour before and after the intervention. DNA was isolated from leukocytes. After a quality control consisting of spectrophotometry and electrophoretic separation, DNA was examined by the methyl ELISA to prove global methylation status.

Results: Depending on the individual subject and exercise intensity changes in global DNA methylation turned out differently. In comparison to the two exercise intensities, the moderate session led to greater methylation of the DNA than the high-intensity session. Global DNA methylation after moderate-intensity cycling revealed a significant difference. 75 % of participants showed an increase in the percentage of methylated DNA. There was no significant change in global DNA methylation after high-intensity cycling.

Conclusion: An interesting review of the literature revealed several other interventional studies that systematically identified a significant impact of acute or chronic exercise on DNA methylation in different tissues. The intensity of exercise can show different levels and effects of DNA methylation due to the analysis method and tissue. However, further in-depth studies are needed to evaluate the effect of exercise interventions on DNA methylation. The used methyl ELISA is a suitable entry-level method for the investigation of changes in DNA methylation.

Keywords: Epigenetic, DNA methylation, endurance exercise, ELISA, peripheral blood

Introduction

The current literature shows generally positive outcomes of physical exercise on human health **[1,2,3,4]**. However, the health benefits of physical exercise are still underrated. Physical exercise shows both preventative and rehabilitative effects regarding disease **[5,6]**.

For adults (18-64 yrs.) the World Health Organization (WHO) recommends an active lifestyle with at least 150 minutes of moderate- intensity aerobic physical activity throughout the week. This is linked to a variety of positive outcomes such as risk reduction of several diseases, for example, breast and colon cancer or stroke **[7,8]**.

Epigenetics includes changes of the nucleosome with no impact on the base sequence of the DNA **[9]**. Positive alterations can be explained by epigenetic factors that are important in salutogenic processes and can be identified as modulators for health-promoting

effects **[10]**. Deactivation or activation of different genes can play a crucial role in these immune reactions. DNA methylation, chromatin remodeling, and histone modification are three major kinds of epigenetic regulations. DNA methylation is the most investigated mechanism of genetic alternations. DNA methylation means the addition or removal of methyl groups (CH_3) by DNA methyltransferases (e.g., DNMT1, DNMT3a, or DNMT3b) at the 5-carbon of the cytosine ring **[11]**. This process leads to the generation of 5-methylcytosine (5-mC). The amount and variation of 5-mC are some of the most important factors in epigenetic modification.

DNA methylation inhibits transcription directly or with help of methyl-CpG-binding proteins (MBPs) recruited co-repressor molecules to silence transcription, while demethylation leads to the opposite **[12]**. Cytosine is very susceptible to methylation, particularly when followed by guanine in 5`-3` direction within the DNA sequence. Such CpG dinucleotides primarily occur in clusters, which are called “CpG islands”. More precisely, these are DNA segments with a length of around 1000 base pairs. They show higher CpG density compared to other parts of the genome and are often unmethylated **[13]**. The majority of gene promoters (approximately 70 %), which form the starting point for RNA polymerase during transcription, are located within CpG islands **[14]**. This relationship establishes the important function of DNA methylation as an epigenetic regulatory mechanism of gene expression due to the influence of various transcription factors. The effect of DNA methylation on gene expression depends on its localization within the genome: In the context of gene promoter regions, the extent of methylation behaves contrary to the activity of gene transcription. DNA methylation of CpG islands results in more difficult DNA reading due to steric hindrance and chromatin compaction. Hypermethylation of CpGs is accompanied by a suppression of transcription, it acts as an “off switch” for gene expression. If the promoter regions, or the CpGs, are hypomethylated, the DNA becomes more accessible to various DNA-binding proteins, which can lead to increased transcription **[15]**.

In the last 20 years, it has been recognized that epigenetics is of great importance for modern medicine, as epigenetic regulatory mechanisms are involved in the development of diseases in various ways. These diseases include various carcinomas, cardiovascular diseases, metabolic diseases such as type 2 diabetes, neurological diseases, and many more. DNA methylation affects the stability of the genome and the balance between tumor suppressor genes and oncogenes and contributes significantly to the development of various cancers (e.g., colon and breast cancer). In general, a decrease in global DNA methylation (= hypomethylation) especially at CpG-depleted regions in combination with site-specific hypermethylation at CpG islands of promoter regions is associated with increased susceptibility to tumor disease. Global hypomethylation has been found in many malignancies. This abnormality was described in the 1980s in association with adenocarcinomas of the colon and lung carcinomas **[16]**. In subsequent years, it has been demonstrated that genome-wide DNA demethylation may contribute to carcinogenesis in several ways: First, hypomethylation of repeat sequences such as LINEs (Long Interspersed Nuclear Elements), which comprise approximately half of the human genome and are normally methylated, leads to their activation and via chromosomal rearrangement processes to chromosomal/genomic instability **[17]**. We expect a positive effect in form of an increase in global DNA methylation under sport. With regard to the promoter specific CpG regions, a site-specific hypomethylation at the promoters is most likely to occur, because reduced methylation could have positive effects such as a higher expression of tumor suppressor genes, e.g., L3MBTL1 **[18]**. A further randomized controlled trial on 276 women, completed supervised aerobic exercise programs for 16 weeks varying a crossed design by intensity (55-65 % vs 75-85 % of VO_{2max}) and duration (40 vs. 20 min per session) led to decreased post-intervention methylation of BRCA1 ($p = 0.01$) **[19]**. Supervised 6-weeks resistance and home-based exercise within colorectal cancer survivors showed methylation changes in 756 CpG sites in promoter regions linked to positive effects (e.g., immune response and transcription) compared to the control group **[20]**. However, this cannot be evaluated here using ELISA.

DNA methylation seems to target gene expression through different influences such as nutrition **[21,22]**, toxic chemicals (e.g., glyphosate **[23]** or exercise **[24,25,26,27]**. Furthermore, exercise can change gene expression through skeletal muscle and metabolic adaptations **[28]**. There is some controversy within the current literature with regard to the link between exercise and DNA methylation. Most of the studies show high heterogeneity in terms of participants, exercise, tissues used for analyses or analysis methods **[29]**.Some of the studies found a link between exercise and the methylation of DNA **[28,30,31,32,33]**. However, there are various discrepancies in the studies, which investigated the connection between exercise and global DNA methylation **[25]**. The current literature of acute exercise studies on the human genome also used muscle biopsies, rather than peripheral blood, as the starting material. In 2012, the first significant results were published. In muscle biopsies from 14 young, physically inactive adults (men and women, age: 25 +/- 1 year) showed decreased genome-wide methylation after an acute exercise intervention (increasing load on bicycle ergometer until fatigue), which depended on the intensity of the exercise. The higher the load, the greater the decrease in DNA methylation after the intervention. In addition, hypomethylation occurred at several genes (PGC-1a, PDK4, and PPAR-d) important for the development of type 2 diabetes at high intensity. At low intensity, there was no significant change in methylation at these genes **[28]**. Thus, Barres et al. (2012) were the first to show that even an acute exercise intervention can intensity-dependent lead to measurable epigenetic modifications in muscle

tissue. While the effects in muscle tissue seem to depend on the load intensity **[28]** a study on subcutaneous adipose tissue samples provided evidence that a person's training status also influences the epigenetic response to exercise. In a more trained state changes in methylation status were more pronounced following an acute intervention **[34]**. The few studies looking at global DNA methylation in leukocytes following an acute exercise intervention showed different results. Robson-Ansley et al. (2014) failed to demonstrate changes in methylation status either immediately following exercise or after 24 hours **[35]**. Nevertheless, they demonstrated a significant link between plasma IL-6- concentration and DNA methylation of 11 genes. Hunter et al. indicated a significant reduction in global DNA methylation in young male cyclists after acute exercise (cycling) with 75 % maximal power output **[36]**. In contrast, Machado et al. (2021) revealed a higher global lymphocyte DNA methylation in three physically exercised groups **[37]**

The aim of this study was to find out if the Methyl-ELISA is capable to detect small changes in global DNA methylation of leukocytes and whether the intensity of acute exercise influences DNA methylation in peripheral blood cells. The test protocol could give interesting insights into the acute influences of exercise on the DNA methylation of leukocytes.

Secondly, the current study can reveal whether there are differences in the DNA methylation of leukocytes after one training session with different intensities, provided the measurement equipment is appropriate. Hence suggestions could be given regarding further in-depth studies in terms of training recommendations.

Materials And Methods

After the approval of the local ethics committee, enrollment of the participants was carried out between November 2018 and January 2019. In total 12 healthy adults participated. The intervention of different exercise intensities was carried out from January 2019 until March 2019. The first tested intensity was high (85 % of individual peak power) and one week later a lower intensity was tested (50 % of individual peak power). The participant group was the same for both exercise interventions in order to observe differences between various intensities. Analysis with the ELISA kit was carried out from June 2019 until October 2019 after collecting the blood samples **(Figure 1)**.

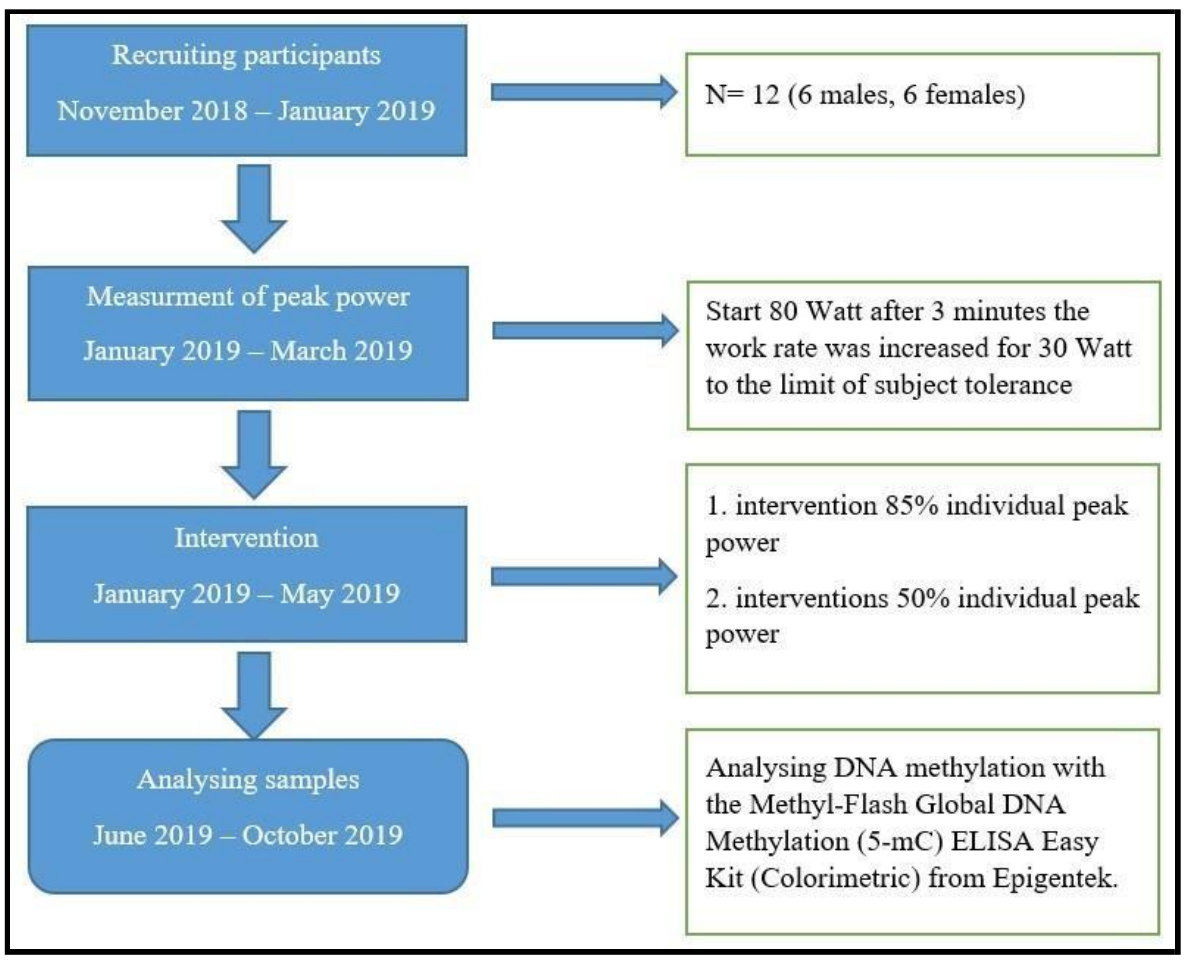

Figure 1: Test protocol

Ethics

According to the ethics regulations of the University of Hildesheim (ethics application of 14.08.2018), all participants signed informed consent of genetic diagnostics law (GenDG) prior to the investigation. The investigation was carried out following the rules of the Declaration of Helsinki.

Participants

Twelve volunteers (female 6; male 6) participated in the study. We included athletic participants with more than 150 minutes. of moderate intensity (3-6 METs, **[38]** aerobic sports per week according to the recommendation of the WHO **[7]**. All participants were between 18 and 28 years old **(Table 1)**. As young individuals show high genomic stability with lower levels of DNA damage and mutations **[39]**, we tried to isolate the effect of physical exercise. Criterions of exclusion were chronic medical conditions, regular intake of drugs, acute or chronic musculoskeletal injuries, smoking, and pregnancy.

Table 1: Anthropometric data of participants (n = 12)

Age	23.75 ± 2.56
Height [cm]	178.17 ± 9.20
Weight [kg]	73.46 ± 16.04
BMI [kg/cm^2]	22.89 ± 2.94
sports/week in minutes	327.5 ± 144.4

Measurement of peak Power

Each participant performed a ramp-type progressive cycle ergometer test using the Polar Ergo fit 401 bicycle ergometer (ERGO-FIT GmbH & Co. KG, Pirmasens, Germany). The participants started at 80 Watt and continued with an increase of 30 Watt after 3 minutes of pedaling respectively until the limit of the individual's capacity was reached. The participants were instructed not to perform any type of vigorous physical activity 48 hours prior to the peak power test and the exercise challenge. The work rate is individualized for all participants according to the following exercise protocol.

Exercise Protocol

Seven days after measurement of peak power, all participants had performed a 6x3 minutes training bout with 85 % of the individual peak power. Between the high-intensity phases, participants had performed 5x3 minutes resting with 25 % of the peak power which summed up to 33 minutes in total. Another seven days later, all participants performed the same test with 50 % of their peak power. For work matched intervention we calculated the exercise duration from 85 % peak power to 50 % peak power. Therefore, all participants had to perform 38 minutes at 50 % of their peak power.

Sample acquisition

One hour before the onset of exercise blood samples (one 7,5 ml tube of Heparin blood) from the antecubital vein were collected for baseline investigation. The same procedure was carried out one hour after the high-intensity and moderate exercise test. Thus, in total four blood samples were taken from each participant **(Figure 2)**.

After collection, the blood samples were frozen at -20°C. For transportation samples were packed in dry ice and sent to the Essen University Hospital by medical transport. After transport, the samples were stored at -20°C again.

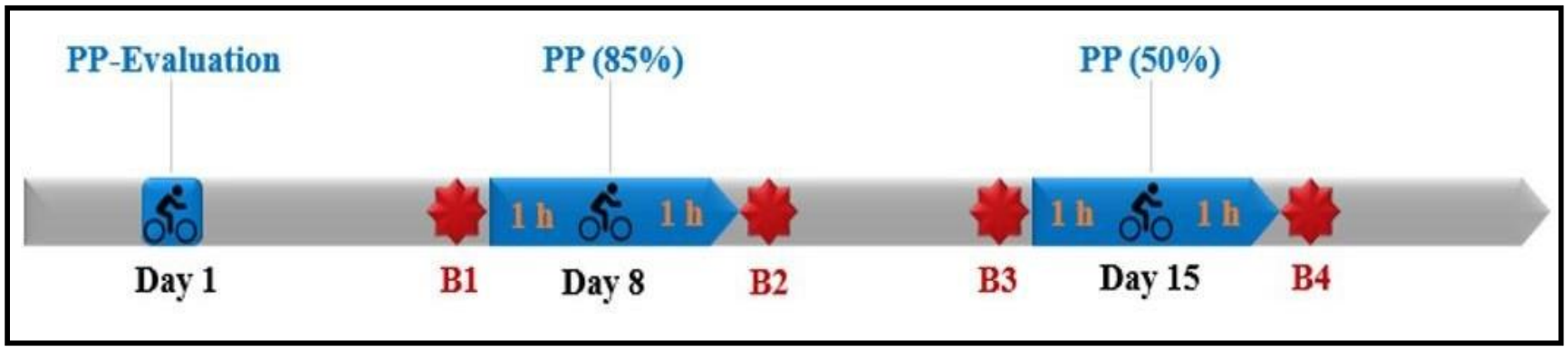

Figure 2: Timeline of the intervention (PP = Peak Power, B = Blood Sample)

DNA extraction

Genomic DNA was extracted from 2 ml peripheral blood of each sample by using the QIAamp DNA Blood Midi Kit (Cat. no.: 51185, QIAGEN©, Hilden, Germany). The spin protocol for the purification of DNA from whole blood (step 1 to 13a, see: „QIAamp DNA Blood Midi/Maxi Handbook 02/2015 pp. 19-21“) was followed. DNA concentration and purity were measured by using the Thermo Scientific™ Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and tested by PCR and gel electrophoresis.

Global methylation analysis in peripheral blood DNA with ELISA

The Methyl Flash™ Global DNA methylation (5-mC) ELISA Easy Kit (Colorimetric) [Base Catalog # P-1030, © Epigenetic Group Inc., Farmingdale, USA] was used in order to determine the global DNA methylation. Based on the ELISA the percentage of single samples was measured. Furthermore, the different samples of each participant could be compared to determine the development of DNA methylation.

In addition to the DNA samples, a standard curve was generated by measuring the negative control and positive controls (0,1 % to 5,0 %). All samples were run in duplicate to avoid incorrect measurement of a well. For subsequent evaluation, the mean of the measurements was used.

Before the assay was started the extracted DNA was diluted with distilled water (c = 25ng/µl). The ELISA could run with the optimal DNA amount of 100ng by adding 4µl to the sample wells.

With help of the binding solution, the input DNA was bound to the assay wells during incubation at 37°C for 60 minutes. After washing the wells, the 5-mC detection complex solution was added to the wells for 50 minutes. This solution includes the 5-mC antibody, to capture the methylated fraction of the DNA, a signal inducer, and an enhancer solution. After another wash step, the developer solution was added to the wells. Within 3-5 minutes, the wells with methylated DNA turned blue. Finally, the enzyme reaction was stopped by adding the stop solution after the 5 % positive control turned deep blue and the color changed from blue to yellow. After 2-5 minutes the absorbance of each well was read by an Infinite 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland) at 450nm.

Data analysis

Based on the measured optical density (OD) the percentage of methylated DNA was calculated. The OD values of the negative control and the positive controls (0.1-5.0 %) served as the basis for creating a standard curve. Linear regression was required to calculate the degree of methylation. The following equation was used to calculate the percentage of methylated DNA:

$$5mc\ \% = \frac{Sample\ OD - NC\ OD\ (average)}{Slope of linear regression * S}\ x\ 100\ \%$$

NC = negative control, S = 100 ng (= DNA input)

Statistical analysis

Normal distribution was performed with the Shapiro-Wilk-Test. Normal distribution was not given for the individual measurement points B1-B4, but the differences „B2-B1 “(85 % PP), „B4-B3 “(50 % PP), „50 % PP-85 %PP” showed a normal distribution without outliers. For comparison of the baseline and the results, the t-test for dependent samples were performed by using the mean of the percentage of methylated DNA of each participant. Significant differences are assumed at $p < 0.05$. All data were analyzed with IBM SPSS Statistics Version 26 (IBM, NY, USA).

Results

The measurement of the positive control (PC) concentration points is shown in a standard curve with a calculated regression line. The slope of this linear regression was part of the equation to calculate the percentage of methylated DNA (see Materials and methods). Thus, the measurement of the PC concentration points had a direct influence on the evaluation of the samples.

There was also a connection between the coefficient of determination and the precision (shown through the intra assay). When the coefficient of determination was near 1. The standard deviation and the coefficient of variation were smaller. These values were calculated from 8 replicates of the same sample (randomly chosen). **Table 2** shows examples of data generated and reported for a representative intra-assay validation experiment. In this example 8 replicates of one DNA sample were tested in two different assays to show the relationship between coefficients of determination and the coefficient of variation.

Table 2: Comparison between two different intra assays at different coefficients of determination (R^2)

	Sample 1	Sample 2
Coefficient of determination (R^2)	0.4769	0.9825
Mean (5-mC%)	2.68%	3.04%
SD	0.498%	0.289%
coefficient of variation CV (%)	18.61%	9.484%

The resultant % CV for the sample was higher when the coefficient of determination tends towards 0. This observation was the reason why we only included ELISA measurements with $R^2 > 0.8$ to ensure the most representative development of the 5-mC.

Global DNA methylation was assessed in peripheral blood DNA from leukocytes of 12 individuals. Global methylation levels determined were 1,46 ± 1,09 of 5-mC % (Minimum: 0,25 %; Maximum: 5,93 %, **see Table 3**).

Table 3: 5-mC%-means (Global-DNA methylation levels) of the participants

5mc%-means	B1	B2 (85%)	B3	B4 (50%)
SM1	0.83%	0.44%	0.40%	0.72%
VL2	0.91%	0.79%	0.70%	1.28%
YK3	0.73%*	0.49%	0.32%	1.16%
CB4	0.79%	1.07%	0.76%	1.41%
BM5	1.18%	0.88%	0.90%	0.83%
DS6	1.12%	1.46%	0.76%	0.65%
BE7	1.68%	1.72%	0.60%	0.62%
JM8	1.28%*	0.54%	0.47%	0.35%
MS9	1.66%	1.94%	2.54%	2.66%
KV10	2.17%	1.35%	2.05%	2.57%
AG11	1.47%	2.02%	2.28%	3.12%*
AP12	3.25%	3.41%	4.18%	5.65%

(Means of participants are calculated from two values); (SM1 – AP12 = Participants; B = Blood Sample) *YK3 B1 only one value, because second value [0.10 %] was probably a measurement error; JM8 B1 only one value, because the second value [5.83 %] was probably a measurement error, * AG11 B4 only one value, because the second value [5.93 %] was probably a measurement error

The participant's reaction seems to be highly individual (range of calculated percentage of methylated DNA was 5.68 %). The changes were inter-and intra-individual to the different stress intensities. It is assumable that various factors could play a role, e.g., nutrition, gender, or even the high variation of the amount of training per week within the group of participants. The Shapiro-Wilk test showed no normal distribution for three of the four approaches (p-values: B1: $0.032 < p = 0.05$; B2: $0.119 > p$; B3: $0.008 < p$; B4: $0.012 < p$). Only approach B2 shows a normal distribution.

Figure 4 shows the distribution of this data in the "Total" box plot and additionally the distribution of the descriptive data for the individual measurement times B1-B4. Although the data for the measurements at times B3 and B4 show significantly greater ranges and variances compared to measurements B2 and B1. The mean value of global DNA methylation for the individual times is in a similar range **(see Table 4)**.

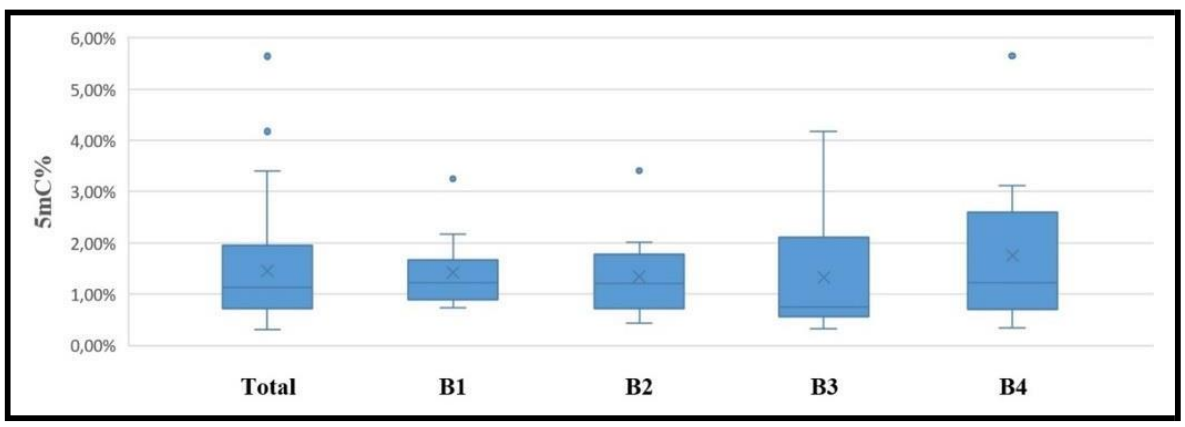

Figure 4: Box plots with global DNA methylation (5-mC %) profiles of the ELISA for different parts of the intervention (B1-B4). The boxplots were calculated in Microsoft Excel by using 5-mC %-Means of **Table 3**

Table 4: Descriptive statistics for different parts of the intervention (B1-B4) and "total" DNA methylation levels.

	N	Range	Minimum	Maximum	Mean	Standard deviation	Variance
B1	12	2.52	0.73	3.25	1.42	0.719	0.517
B2	12	2.97	0.44	3.41	1.34	0.854	0.730
B3	12	3.86	0.32	4.18	1.33	1.183	1.400
B4	12	5.30	0.35	5.65	1.75	1.526	2.330
Total	48	5.33	0.32	5.65	1.46	1.093	1.195

Table 5 shows the two dependent means for each participant. The high-intensity cycling (B1 → B2 at 85 % peak power) led to a decrease in DNA methylation in the range of 0.12 % - 0.82 % in 6 participants and to an increase in DNA methylation in the range of 0.04 % - 0.55 % in 6 participants. On average the global DNA methylation decreased by 0.08 % (SD: ± 0.43 %, Var: 0.19). The moderate-intensity cycling (B3 → B4 at 50 % peak power) led to a decrease in DNA methylation in the range of 0.07 % - 0.12 % in 3 participants and to an increase in DNA methylation in the range of 0.02 % - 0.84 % in 9 participants. On average global DNA methylation increase by 0.42 % (SD: ± 0.48 %, Var: 0.24).

Table 5: Differences in DNA methylation levels due to the different training intensities (85 % versus 50 % peak power)

5mc%-means	B2 -B1 [85% Peak Power]	B4 -B3 [50% Peak Power]	Difference 50% PP – 85% PP
SM1	- 0.39%	+ 0.32%	+ 0.71%
VL2	- 0.12%	+ 0.58%	+ 0.70%
YK3	- 0.24%	+ 0.84%	+ 1.08%
CB4	+ 0.28%	+ 0.65%	+ 0.37%
BM5	- 0.30%	- 0.07%	+ 0.23%
DS6	+ 0.34%	- 0.11%	- 0.45%
BE7	+ 0.04%	+ 0.02%	- 0.02%
JM8	- 0.74%	- 0.12%	+ 0.62%
MS9	+ 0.28%	+ 0.12%	- 0.16%
KV10	- 0.82%	+ 0.52%	+ 1.34%
AG11	+ 0.55%	+ 0.84%	+ 0.29%
AP12	+ 0.16%	+ 1.47%	+ 1.31%
Gesamt	MV: - 0.08% SD: 0.43; Var: 0.19	MV: + 0.42% SD: 0.48; Var: 0.24	MV: + 0.50% SD: 0.57; Var: 0.32

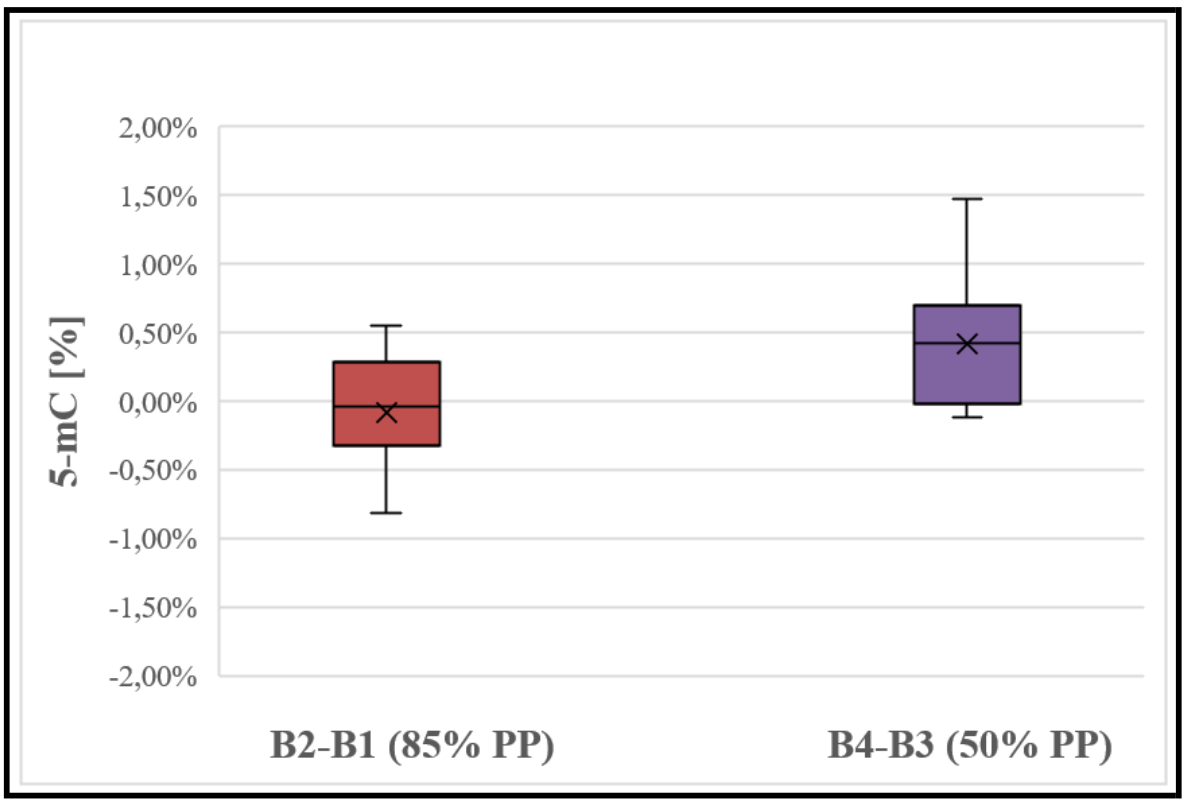

Figure 5: Distribution of the differences in the DNA methylation level before and after the respective training load in form of box plots (calculated in Microsoft Excel by using data of Table V.)

The values B2-B1 (85 % PP) and B4-B3 (50 % PP), which reflect the effects of the differently intensive training sessions, showed a normal distribution each (85 % PP: $p = 0.668 > 0.05$; 50 % PP: $p = 0.258 > 0.05$). The comparison of the two intensities [(B4-B3–) - (B2-B1)] **see Figure 5**, also shows a normal distribution ($p = 0.859 > 0.05$). Therefore, the t-test for dependent samples was used to evaluate the results.

To examine if the exercise affects DNA methylation, we measured the effect size by using Cohen´s D. Cohen`s D can measure the strength of the solved treatment by comparing the means of both groups. In this pilot study, well-trained individuals were included. This could be a possible reason for the low effect size in our data. According to Fabre and associates (2018), this could be a possible reason for the low effect size within the current data.

The following final results can be drawn from the t-test and the additional calculation of the effect size d: There are no significant results for the comparison of the percentage of methylated DNA before (B1) and after (B2) high intensity (85 % PP) cycling (p =

0.535, *d* = -0.185). However, the comparison of percentages of methylated DNA before (B3) and after (B4) the moderate intensity (50 % PP) revealed a significant difference (p = 0.012, *d* = 0.8647). According to Cohen´s D, there is a strong effect of moderate exposure on DNA methylation. Finally, the comparison of both interventions showed significant (p = 0,011) higher percentages of methylated DNA after the moderate workout. According to Cohen´s D, this also shows a strong effect (*d* = 0.8845).

Discussion

The main purpose of the current methodological study was to analyze and compare changes of global DNA methylation with the Methyl-ELISA of Epigenetic© after different intensities in young healthy participants. It should be checked whether the ELISA can detect the small changes in the methylation status which are assumed **[40,41]**. The used ELISA has several advantages. It delivers comparable results within a few hours with no cross-reactivity to unmethylated cytosine or hydroxy methylated cytosine. Assuming a regression degree close to 1 the intra-assay suggests good reproducibility of the measurement results. The coefficient of variation was below 10 % in this case **(see Table 2)**. Furthermore, the probability of measurement errors was decreased by performing duplicate measurements of each individual sample. The previous quality control of the DNA using spectrophotometry and gel electrophoresis prevented the ELISA test from being falsified as a result of insufficient DNA concentrations or DNA fragmentation.

The other options for analyzing DNA methylation are diverse and include methods to interrogate global DNA methylation. Gene-specific analysis of DNA methylation (e.g., pyrosequencing, Methylation-Specific qPCR) and sequencing Bisulfite Converted DNA **[42]**. In addition to ELISA other methods are suitable for determining global DNA methylation, such as the LUMA (Luminometric Methylation Assay) and the examination of repetitive sequences such as Alu repeats or LINE repeats. The latter is examined by PCR amplification following bisulfite treatment. **[43]**. The LUMA is based on DNA cleavage using methylation-sensitive restriction enzymes followed by pyrosequencing to quantify global DNA methylation **[44]**. Both methods offer no advantages compared to the ELISA. The ELISA maps the entire genome, requires a smaller amount of sample DNA and does not require bisulfite modification like the investigation of "repeat sequences".

Despite the named advantages, performing the ELISA also has limitations such as the preparation and measurement of the positive control series. The slope of linear regression inside the standard curve **(see Figure 3)** is a part of the formula for calculating the DNA degree of methylation (see data analysis). In addition, the precision of an ELISA depends on the coefficient of determination R^2 and thus also on the positive control series. With an $R^2 > 0.8$ a standard was set for the most precise ELISA measurements, however, there is still room for small deviations in the accuracy of the methylation data in the permitted range of 0.8-1. Even with an almost perfect standard curve (R2 close to 1), measurements from the same samples deviate by approx. 10 % (CV % = 9.484 % with R^2 from 0.9825, **see Table 2**). Small changes, as can be seen in test person BE7 (B1: 1.68 % → 85 % PP → B2: 1.72 % | B3: 0.60 % → 50 % PP → B4: 0.62 %, **see Table 3**), cannot be evaluated due to the existing degree of variation. Another disadvantage is the color reaction of the ELISA in the wells: The blue coloration of the wells (enzyme reaction by the developer solution) should be stopped by adding the stop solution at the point “when the color in the 5 % PC wells turns deep blue” (Step 4e, page 8 “Methyl Flash ™ Global DNA Methylation 5 mC ELISA Easy Kit [Basic Catalog # P-1030]). This instruction is not specific. The time to stop the enzyme reaction depends on the estimated color development (blue) in the 5 % PC well. This subjective assessment when the 5 % PC wells turn deep blue is highly examiner dependent and can lead to deviations in the interassay. We added the stop solution 5 minutes +/- 30 seconds after adding the developer solution.

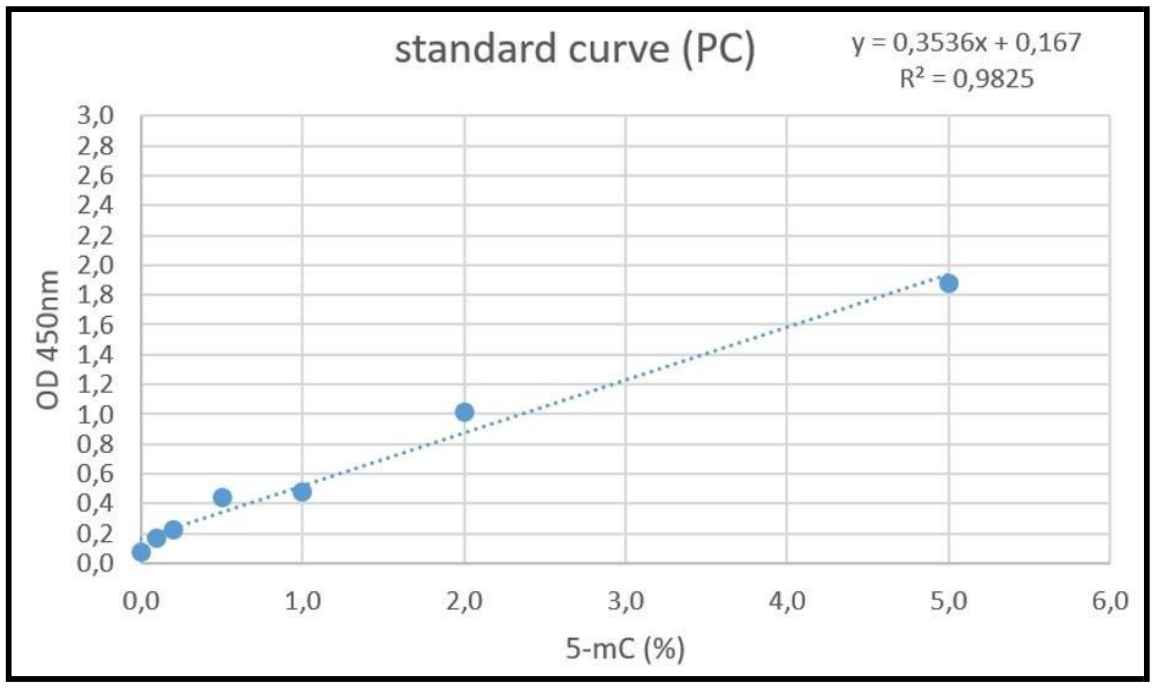

Figure 3: Example of a standard curve

The results of the physical intervention clearly demonstrated an acute change in the methylation status in the test participants. The high intensity showed no significant change (p = 0.535) in DNA methylation status in the 12 participants. In contrast, the moderate-intensity showed a significant increase (p = 0.012) in the methylation status. In 75 % of the participants, moderate intensity led to a rise in global DNA methylation.

The Cohen´s D values show a difference (85 % PP: *d* = -0.185; 50 %: *d* = 0.8647), however, this analysis option needs to be interpreted with caution as only 12 participants can be included in the statistics. Performing the same study protocol with a control group of untrained participants (< 150 minutes sports/week) could possibly lead to higher effects on DNA methylation as those persons would begin at a lower level with less or no adaptation to sporting stimuli in contrast to the participants of the current study.

With regard to the small cohort and the selection of the test group, these results should rather be considered as a trend and need further in-depth research. The selected study design of the pilot project did not include a control group which should be included in future investigations. Nevertheless, against the background of the current study situation, some pointers can be found on the acute adaptation of DNA methylation in leukocytes after an exercise intervention. The proven increase in global DNA methylation due to moderate exposure could be a hint that even a single acute training session has the potential for a cancer-preventive effect on a molecular level as global hypomethylation is a marker in tumorigenesis (see introduction) and in the aging process **[45]**. However, A comparable publication by Robson-Ansley et al. (2014) on 8 young adult men could not show any significant changes in the methylome as part of an acute endurance intervention **[35]**. While there are still no significant results in terms of DNA methylation. Radom-Aizek et al. (2014) show changes in several micro-RNAs after a 20-minutes intervention on a bicycle ergometer. While Hunter et al. (2019) revealed a decrease in global DNA methylation, Machado et al. (2021) exhibited an increase in global DNA methylation after exercise.

This pilot study provides further evidence that acute exercise can change the human epigenome. Even the hitherto existing non-significant results of both the current and previous investigations are worth to be checked more thoroughly using larger sample sizes in future studies.

In comparison, the moderate exposure level appeared to have a stronger influence on DNA methylation. This confirms the current hypothesis that the intensity of physical activity also seems to play a role in influencing epigenetic regulation. Barres et al. (2012) postulated that the change in DNA methylation is proportional to the intensity of exercise **[28]**. However, their results are based on muscle tissue instead of peripheral blood. The changes in DNA methylation are hard to be compared as the adaptation of DNA methylation is different depending on the tissue **[47,48]**. Nitert and Rönn et al. (2012) established a significant correlation between physical activity and the methylation of various markers associated with type 2 diabetes mellitus and obesity using a genome-wide investigation of the same patient collective. A monitored six month endurance training (3 hours/week) led to hypomethylation of most genes measured in biopsied muscle tissue, whereas hypermethylation of several genes was found in adipose tissue **[48]**.

It remains unclear whether only the intensity or the duration influences the effect. A difference in DNA methylation between acute and chronic exposure cannot be excluded.

The global DNA methylation pattern in total **(see Figure 4)** showed a range of different values [1.46 ± 1.09 of 5-mC % (0.25% - 5.81 %)]. The extent of the changes was different for each test person as shown by the differences in initial levels of DNA methylation **(see Table 3)**. Within other studies examined isolated DNA from peripheral blood displayed similar results **[40,41]**. This could be explained by the abundance of confounders. Common examples of these factors are sex, age, BMI, and smoking. For instance, inflammation is known to promote DNA hypermethylation, making it an important confounder **[49]**.

Manzardo et. al. (2016) determined global DNA methylation levels 2.2 ± 1.1 % (0.06–5.6 %) in their study about global methylation in Prader-Willi-Syndrome after extracting DNA from peripheral blood of 91 adults. Tog hill et al. (2018) assessed global genomic DNA methylation in peripheral blood DNA of 185 individuals (patients with an abdominal aortic aneurysm and controls). Including all samples (control, small AAA, and large AAA) they determined global DNA methylation levels ≈ of 0.1 – 3.5 % and showed a linear relationship between AAA size and global DNA methylation. The used ELISA does not seem to be responsible for the wide range of measured DNA methylation levels. A limitation of all studies is a large number of confounders, such as gender, age, BMI, lifestyle factors (nicotine consumption, diet, etc.), and various environmental factors that also seem to have an effect on the epigenome **[50]**.

The chosen ELISA of Epigenetic© seems not to be able to prove small differences (< 10 %) in global DNA methylation as even a well-performed intra-assay CV % test leads to a variation of approx. 10 % **(see Table 2)**. The ELISA method seems to be suitable for the detection of larger global DNA methylation differences, like the correlation between global DNA methylation and the AAA size **[41]**, and its course in long-term interventions.

In order to verify the trend of the ELISA results, this method needs to be compared to other approaches for analyzing DNA methylation prospectively.

Conclusion

The current research in this area is small and heterogeneous with investigations to different species (for example zebrafish, mouse, human, …) and different starting materials (blood, different tissues e.g., muscle cells, gem cells). Comparison to studies that have worked with peripheral blood shows that results of the percentage of methylated DNA are similar.

The given data showed different outcomes of DNA methylation after moderate or high-intensity exercise. After calculating effect sizes from Cohen's d´ it could not be determined if these effects were merely resulting from the performed exercise. The carried-out exercise intervention with a high intensity showed no increase in DNA methylation, whereas the moderate-intensity led to an increase in DNA methylation.

The combination of all observations and results suggests that the performed ELISA of EpiGentek© may show minor changes in the methylation status. The interpretation of these detected changes is limited because changes in the degree of methylation of up to 10 % cannot be delineated from the intra-assay CV %. Even if the results can only be assessed to a limited extent, the ELISA has the advantage that it is possible to receive comparable values of global DNA methylation within a few hours. Furthermore, the costs of the ELISA are comparatively low and it is an easy-to-use method for analyzing DNA methylation.

Due to its essential advantages, the methyl ELISA from EpiGentek© is a suitable entry-level method for the investigation of changes in DNA methylation.

Informed consent: Informed consent was obtained.

Data Availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations: 5-mC: 5-methylcytosine; AAA: abdominal aortic aneurysms; AML: acute myeloid leukemia; CpG: 5'-cytosine-phosphate-guanine-3'; CV %: coefficient of variation; DNA: deoxyribonucleic acid; DNMT: DNA methyltransferase; ELISA: Enzyme-linked Immunosorbent Assay; L3MBTL1: lethal(3)malignant brain tumor-like protein, isoform 1; MET: metabolic equivalent; MV: mean value; OD: optical density; PC: positive control; PP: peak power; SD: standard deviation; Var: variance; WHO: World Health Organization

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