THE COMPARATIVE METHYLOME ANALYSIS AFTER ACUTE ENDURANCE EXERCISE

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Table of contents:

Background **2**

Hypothesis and Objective **2-3**

Specific aims and Experimental plan **3**

Methodology **4-5**

Results, Discussion and Limitations **6-9**

Reference list **10-11**

Appendix **12-16**

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Background

One of the processes of epigenetic modifications is DNA methylation. It is an epigenetic mechanism in which a methyl group is added to a cytosine's C5 position to produce 5-methylcytosine [1].
The most prevalent sites for DNA methylation are CpG sites, or cytosine-guanine (C-G) base pairs. CpG islands are CpG-rich regions found in gene regulatory regions such as the promoter and enhancer. The bulk of CpG sites in humans (70-80%) are methylated [2, 3]. When methylation is present or increases (hypermethylation), transcription factors are unable to bind to specific areas of the gene, and gene expression is reduced [4]. Conversely, loss of methyl groups (hypomethylation) at specific sites might enable/increase gene expression.

Cancer risk [5, diabetes [6, CVDs [7], major depressive disorder [8, autoimmune disorders [9], and aging [10] have all been related to changes in DNA methylation at specific genomic locations.

Exercise has been shown to affect DNA methylation profiles in blood, muscle, and adipose tissue in earlier investigations [11, 12, 13, 14].

The effects of various forms of exercise on DNA methylation were investigated. In humans, aerobic exercise training alters the methylome of skeletal muscle, hypomethylating the genome more frequently in patients with type 2 diabetes and obesity [15, 16].

Eight young (21.1 2.2 years) males undertook 8 weeks of supervised, thrice-weekly resistance exercise training consisting of three sets of 8–12 repetitions with a weight comparable to 80% of 1RM, as well as changes in genome-wide DNA methylation and gene expression [13].

Furthermore, the duration of activities varies amongst research. Some research looked at chronic exercise regimes, while others looked at acute exercise protocols. In one research, two different sport-relevant high-intensity running protocols were used: Exercises: 2) straight line vs. 1) change of direction (COD) (ST). Researchers took skeletal muscle samples 44 from the vastus lateralis 30 minutes and 24 hours after exercise. Exercise alone caused substantial alterations in the 50 methylome 30 minutes and 24 hours after exercise in both conditions, particularly in the MAPK, AMPK, and axon guidance 51 pathways [12].

Only a few studies, however, have looked at DNA methylation across the genome in healthy human blood after acute endurance exercise.

Premise of the hypothesis

Finding novel DNA methylation modifications in response to physical activity might assist to avoid the onset of some illnesses and better understand how physical activity affects DNA methylation.

Hypothesis

This study was designed to assess the hypothesis that acute endurance exercise can affect DNA methylation in human blood in time-specific manner.

Objective

The purpose of this work was to look at changes in DNA methylation across the genome using a genome-wide search for enriched gene ontologies, pathways, and chromosomal location in human blood before, during, and after acute endurance exercise. Furthermore, to look at the changes in DNA methylation between persons who are active for a long time, moderately active, and inactive.

Specific aims

1. Getting an ethical approval and recruiting volunteers
2. Pretesting assessment and performance of exercise protocol.
3. Blood collection
4. DNA extraction and DNA methylation analysis
5. Statistical analysis of findings

Experimental plan

1. Obtaining the Ethical Committee's clearance and recruiting volunteers.
   a) The Ethical Committee of Nazarbayev University granted clearance for this study.
   b) Volunteers were chosen from a healthy male population with no health issues, as well as professional athletes.
   c) This study included six elite athletes, six people who engage in moderate daily exercise, and six people who are inactive.
   d) All participants signed the [Appendix 3] informed consent form.
2. Assessment and execution of the workout regimen prior to pre-testing.
   a) Participants completed the IPAQ Questionnaire (International Physical Activity Questionnaire) before the experiment to determine their level of physical activity [17] [Appendix 2].
   b) After a 5-minute warm-up, the experimental protocol consisted of running or walking on a treadmill for 1 hour [18].

3. Obtaining blood

Blood was taken from veins before exercise (0), four hours after exercise, and twenty-four hours after exercise.

4. Isolation of DNA and methylation study of DNA

DNA will be obtained from blood samples using the sodium acidic precipitation technique [19], and samples will be forwarded to DNA methylation analysis.

5.Findings statistical analysis

The test will be a one-way ANOVA.

Methodology

- Getting an approval from the Ethical Committee of Nazarbayev University and recruitment of volunteers [Appendix 1].
- Pretesting assessment

IPAQ questionnaire was presented to participants. According to results of IPAQ questionnaire participants were divided to 3 groups: elite athletes, moderately trained and sedentary group.

• Blood collection and exercise routine

The exercise routine was carried out by moderately trained and inactive groups.

The experimental methodology included a warm-up and one hour of jogging or walking on a treadmill at 65-75 percent of maximum heart rate [18].

For genome-wide DNA methylation study, blood samples were taken from veins before, during, and after the exercise (0, 4, and 24 hours). Only one time was blood taken from elite athletes' groups (0). Before DNA isolation, blood samples were collected in vacutainers containing EDTA (Ethylenediaminetetraacetic acid) and kept at 2-6°C.

Table 1 Blood collection schedule

	Elite (n=6)	**Regularly trained (n=6)**	**Sedentary (n=6)**
Before (0-hour sample)	+	+	+
4-hour sample	-	+	+
24-hour sample	-	+	+

- DNA isolation and bisulfite conversion, DNA methylation analysis

a) DNA isolation was carried out according to a methodology published previously [19].

1. Place 20 l of QIAGEN Protease (or proteinase K) in the bottom of a 1.5 ml microcentrifuge tube.

2. Filling the microcentrifuge tube with 200 l of sample Using 200 l whole blood, plasma, serum, buffy coat, or bodily fluids, or 5 x 106 lymphocytes in 200 l PBS

3. Pour 200 l of Buffer AL into the sample. 15 seconds of pulse-vortexing mixing.

4. Incubating for 10 minutes at 56°C.

5. Remove drips from the inside of the lid by centrifuging the 1.5 ml microcentrifuge tube briefly.

6. Adding 200 l ethanol (96–100%) to the sample and pulse-vortexing for 15 seconds to mix it again. After mixing, centrifuge the 1.5 ml microcentrifuge tube for a few seconds to remove any remaining drips from the inside of the lid.

7. Using a 2 ml collection tube, carefully add the liquid from step 6 to the QIAamp Mini spin column without soaking the rim. Closing the cap and centrifuging for 1 minute at 6000 x g (8000 rpm). Using a clean 2 ml collection tube, place the QIAamp Mini spin column and discard the filtrate tube.

8. Without wetting the rim, carefully open the QIAamp Mini spin column and add 500 l Buffer AW1. Close the cover and centrifuge for 1 minute at 6000 x g (8000 rpm). Remove the QIAamp Mini spin column from the collection tube holding the filtrate and place it in a clean 2 ml collection tube.

9. Without wetting the rim, carefully open the QIAamp Mini spin column and add 500 l Buffer AW2. Close the lid and centrifuge for 3 minutes at maximum speed (20,000 x g; 14,000 rpm).

10. Using a clean 1.5 ml microcentrifuge tube, place the QIAamp Mini spin column and discard the collection tube holding the filtrate. Open the QIAamp Mini spin column carefully and fill it with 200 l Buffer AE or distilled water. Incubate for 1 minute at ambient temperature (15–25°C), then centrifuge for 1 minute at 6000 x g (8000 rpm).

b) Whole Genome Sequencing was planned to be performed.

Illumina Whole Genome Bisulfite-treated Library will be used to explore methylation patterns [20].

- Statistical analysis of findings

To assess changes in DNA methylation before and after endurance exercise, a one-way ANOVA analysis will be used. Furthermore, before the exercise, the DNA methylation patterns of top athletes will be compared to those of a moderately trained and inactive group.

Results

Blood samples from 15 participants were collected after obtaining informed consent. Every participant filled out IPAQ questionnaire and underwent InBody 770 measurement of body composition. Participants from sedentary and moderate activity groups performed exercise protocol of walking or running on treadmill on 65-75% of peak heart rate.

IPAQ questionnaire calculates energy expenditure per week. 1 MET (Metabolic equivalent of task) is the rate of energy expenditure while at rest. Summarizing sections like work, leisure time activity, transportation, and gardening. We calculated MET/minutes per week for every participant. Then we separated participants in three groups according to MET/minutes per week, but not according to IPAQ questionnaire grading system. The reason for that was overestimation of activity level by participants, because of subjective nature of questionnaire.

Thus, participants were separated in three groups: Sedentary, Moderate activity and Athletes. Average MET/minutes per week were 1752, 4314 and 14429, respectively.

Table 2 Sedentary group measurements

	MET/minutes Per week	IPAQ group	weight (kg)	height (cm)	BMI	body fat percentage	age	peak heart rate	65-75% heart rate	m. heart rate
P7	1298	moderate	80	188	22,8	17,9	23	197	128-148	148
P8	2483	moderate	63	178	19,9	17,3	29	191	124-143	124
P14	2992	moderate	90	175	29,3	33,4	25	195	126-146	135
P15	990	moderate	97	188	27,7	26,6	28	192	124-144	134
P16	996	moderate	93	182	28,1	31,5	28	192	124-144	134
mean	1752		84,6	182,2	25,56	25,34	26,6	193,4		135

**MET/minutes per week,*

Table 3 DNA yield and purity. Sedentary group

	DNA 0-hour yield (µg)	Purity 260/280	DNA yield 4 hrs (µg)	Purity 260/280	DNA yield 24 hrs (µg)	Purity 260/280
P7	3,9	2,02	1,62	1,82	2,08	2,05
P8	3,72	1,94	5,08	1,8	5,32	1,96
P14	4,38	1,98	8,54	1,86	4,58	1,91
P15	5,86	1,86	4,72	1,82	5,02	1,98
P16	3,7	1,74	2,8	2,02	2,24	2,19

Sedentary group consisted of 5 participants with mean BMI (Body mass index) = 25.56 and Body fat percentage of 25.34. All participants reached their targeted heart rate throughout exercise protocol performance and mean heart rate was between 65 and 75%.

Table 4 Moderate activity group measurements

	MET/minutes per week	IPAQ group	weight (kg)	height (cm)	BMI	body fat percentage	age	peak heart rate	65-75% heart rate	m. heart rate
P1	4911	high	70	173	23,5	19	26	194	126-146	126

P2	4688	high	78	180	24,2	20,2	24	196	127-147	130
P3	4110	high	75	178	23,8	21,5	26	194	126-146	126
P5	3280	high	57	171	19,6	10,2	20	200	130-150	130
P6	3705	high	51	181	15,6	5,6	20	201	131-151	131
P17	5187	high	89	181	27	23,9	29	191	124-143	131
mean	4314		70	177	22,2	16,7	24	196		129

Table 5 DNA yield and purity. Moderate activity group

	DNA 0-hour yield (µg)	**Purity 260/280**	**DNA yield 4 hrs (µg)**	**Purity 260/280**	**DNA yield 24 hrs (µg)**	**Purity 260/280**
P1	1,68	2,11	4,9	1,87	4,48	1,79
P2	3	1,79	3,76	1,91	5,48	2,05
P3	3,94	1,76	4,48	1,77	5,94	1,86
P5	4,28	1,89	4,66	1,86	3,34	1,81
P6	3,54	1,88	3,74	1,87	3,84	1,85

Moderate activity group had mean MET/minutes per week of 4314, BMI 22.2 and Body fat percentage of 16.7.

Table 6 Athletes group measurements

	MET/minutes per week	**weight**	**height**	**BMI**	**body fat percentage**	**age**
P9	15707	68	178	21,6	8,7	20
P10	9828	67	176	21,8	7,9	21
P11	16746	72	183	21,5	6,4	21
P12	9960	67	178	21,2	14,4	20
P13	19903	66	173	22,1	9,1	23
mean	14429	68	177	21,6	9,3	21

Table 7 DNA yield and purity. Athletes group

	DNA yield (µg)	**Purity 260/280**
P9	3,3	1,88
P10	1,76	1,91
P11	4,46	1,82
P12	2,64	1,98
P13	2,32	1,83

Group of Elite Athletes achieved average MET/minutes per week of 14429, BMI 21.6 and Body fat percentage of 9.3.

Blood samples from all participants were stored in 2-6°C and DNA isolation was performed from every sample. Yield and purity of DNA was acceptable for methylation analysis in Macrogen Laboratory. Lowest yield per sample was 1.62 µg and highest 8.54 µg

Table 8 MET/minutes comparison

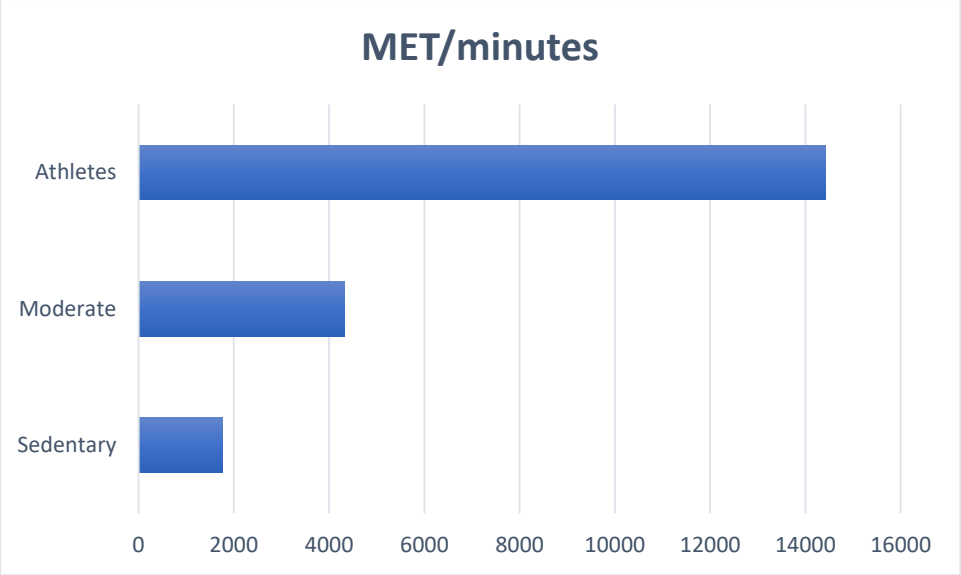
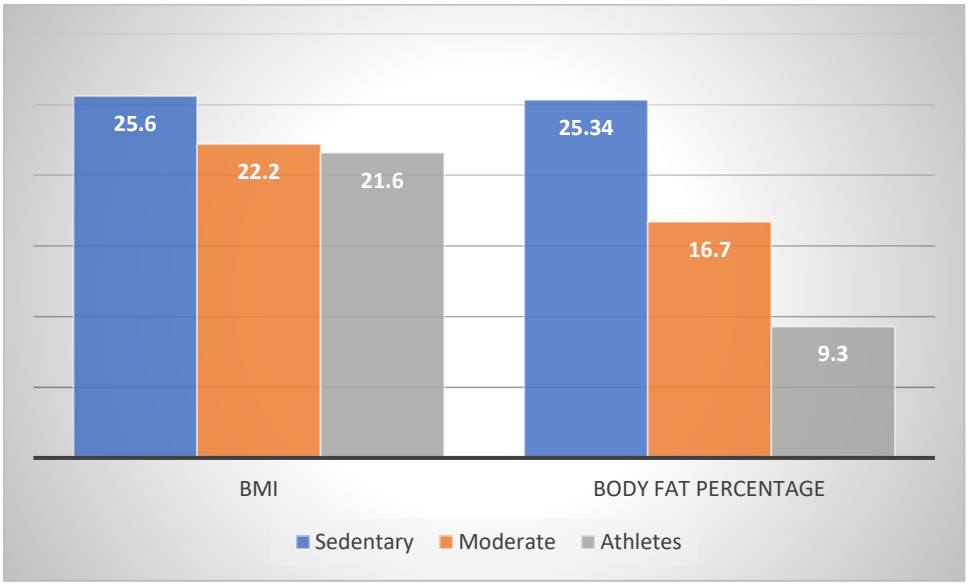


Table 9 BMI and Body fat percentage group comparison



Discussion

At the end of the study blood samples were collected and DNA was isolated from three different physical activity groups. Accuracy of separating in groups not according to standard IPAQ interpretation could be assessed by looking at average MET/minutes per week. Sedentary group had almost half of MET/minutes per week of average in Moderate group. Athletes had 3 times more MET/minutes per week than Moderate group which can be explained by tremendous levels of work done by athletes.

Results of body composition measurement were similar: Body fat percentage of athletes was 9.3 which is less than that of Moderate group by 2 times and Sedentary group by 3 times.

Previous studies that look at DNA methylation data usually were prescribing long-term physical activity [13] or were looking for methylation is specific regions that relate to gene expression [5].

Difference of our study is that we used short term physical activity with targeted range of heartbeat per minute, that was described previously, to assess the effect of exercise on DNA methylation in blood. Furthermore, we collected blood from three different groups of daily physical activity, and we can look at differences in DNA methylation before the exercise performance.

Limitations

Limitation of the study was small sample size. One of the reasons for that was cost of the methylation analysis.

Furthermore, it was hard to finish the study on time because of the COVID-19 pandemic situation and situation in Almaty city which postponed the research activity for mor than a month.

Another limitation was that participants were recruited only from male population and that is why sample cannot be representative of all population.

Finally, timing of the final part of research coincided with Muslim month of Ramadan when part of participants was fasting, which can potentially affect results of the study.

Potential follow-up researchers can consider limitations of this study and plan to: recruit more volunteers, recruit from female population, plan and be flexible with timing, take into account cultural features as fasting of participants during particular time of the year.

Conclusion

We did not achieve goals of our study because of different reasons as mentioned above. Nevertheless, we did half of the planned research, which is recruiting participants, getting answers for IPAQ questionnaire, anthropometric measurements, performance of exercise, collection of blood and extraction of DNA.

Acknowledgements

Thanks to supervisor: Syed Ali, PhD

Thanks to co-supervisor: Burkitkan Akbay, PhD

Thanks to: Maxim Kriat and Kazakhstan Triathlon Federation for cooperation in research

Thanks to: Zhandos Bissarinov, MD, for donating blood for practice of DNA extraction

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Appendix 1
Ethical Approval

NAZARBAYEV UNIVERSITY
SCHOOL OF MEDICINE

Nur-Sultan, 25 November 2021

RE: Decision on the project "Comparative methylome analysis after acute endurance exercise" – OCT#03

The integrated student-driven project **"Comparative methylome analysis after acute endurance exercise"** having **Bauyrzhan Toktarbay,** as Principal Investigator, and **Dr Syed Ali** as Research Advisor, is part of the Master in Sport Medicine and Rehabilitation curriculum.

The above-mentioned student-driven project was evaluated by the NUSOM-IREC (Nazarbayev University School of Medicine – Institutional Research Ethics Committee) as "Expedite" Ethical review.

*This is to inform you that the aforementioned research has been **approved from the NUSOM- IREC as Application NUSOM-IREC-2021-OCT#03** on November 25th, 2021.*

Prof. Alessandro Salustri
Chair of NUSOM-IREC

Appendix 2

IPAQ Questionnaire

#	Question	Answer	
1	Do you currently have a job or do any unpaid work outside your home?	yes	no
2	During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing upstairs as part of your work?	_days/ week	

3	How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?	_____ hours per day _____ minutes per day
4	Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work. Please do not include walking.	_____ days per week
5	How much time did you usually spend on one of those days doing moderate physical activities as part of your work?	_____ hours per day _____ minutes per day
6	During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.	_____ days per week

7	How much time did you usually spend on one of those days walking as part of your work?	_____ hours per day minutes per day
8	During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?	_____ days per week
9	How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle? Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.	_____ hours per day _____ minutes per day
10	During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?	_____ days per week
11	How much time did you usually spend on one of those days to bicycle from place to place?	_____ hours per day minutes per day
12	During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?	_____ days per week
13	How much time did you usually spend on one of those days walking from place to place?	_____ hours per day minutes per day
14	Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?	_____ days per week

15	How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?	_____ hours per day _____ minutes per day
16	Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?	_____ days per week
17	How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?	_____ hours per day _____ minutes per day
18	Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?	_____ days per week
19	How much time did you usually spend on one of those days doing moderate physical activities inside your home?	_____ hours per day _____ minutes per day
20	Not counting any walking, you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?	_____ days per week
21	How much time did you usually spend on one of those days walking in your leisure time?	_____ hours per day _____ minutes per day
22	Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?	_____ days per week
23	How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?	_____ hours per day _____ minutes per day
24	Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?	_____ days per week
25	How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?	_____ hours per day _____ minutes per day
26	During the last 7 days, how much time did you usually spend sitting on a weekday?	_____ hours per day _____ minutes per day
27	During the last 7 days, how much time did you usually spend sitting on a weekend day?	_____ hours per day minutes per day

Appendix 3

Written Informed Consent Form (Moderately Active and Sedentary group)

Introduction. You are invited to participate in a research study entitled The Comparative Methylome Analysis of short- and long-term effects of endurance training. This study will compare epigenetic changes in DNA methylation of sedentary, moderately active people and elite athletes. Research will help to identify how endurance exercise can affect DNA methylation and potentially help to predict and prevent manifestation on different diseases. For this study you will need to go through exercise protocol of 1-hour moderate intensity treadmill and give 3 blood samples (before, 4 hours and 24 hours after exercise).

Procedures. *The interview will be conducted to collect data about physical activity status. The Questionnaires comprises a 27 short-answer questions and can be administered through either the in-person interview or online interview through zoom platform (depending on epidemiological situation in Nur-Sultan). The purpose of this questionnaire is to provide comparable data on health–related physical activity.* This *interview* will take approximately 10 minutes to complete.

The experimental groups, excluding triathletes, will receive endurance exercise that consist of 1-hour treadmill.

Blood collection will be conducted in Nazarbayev University Athletic Center by medical practitioner. The blood sample will be taken 3 times: once before exercise, and then 4- and 24- hours post-exercise. Around 6 ml of blood will be taken. This procedure will take approximately 5 minutes to complete. In the case of triathletes, only one blood sample will be collected from each athlete.

Risks. The potential risks of participating in this study are:
The intensity of endurance exercise will be controlled by pulsometer to around 110-130 bp/min in order avoid overstraining of body. Also, warm up procedure will be organised for injury prevention.
The blood collection considered to be a minimal risk procedure as it could potentially cause some sense of discomfort.

Benefits. *This study will help to provide answers for following questions: Does single exercise provide enough stimuli for DNA methylation? How specific*

training affects the body conditions on molecular level and do the up- and methylation patterns contribute to better health state or athletic performance?

The investigations will result in better understanding how different exercise and time of modulate human methylome. This could be beneficial in exercise prescription management.

Compensation. No tangible compensation will be given. A copy of the research results will be available at the conclusion of the study.

Confidentiality & Privacy. Any information that is obtained during this study will be kept confidential to the extent permitted by the law. All efforts, within reason, will be made to keep your personal information in your research record confidential but total confidentiality cannot be guaranteed. *A participant's study information will not be released without the written permission of the participant. All study-related information will be stored securely at the study site. All participant information will be stored in locked file cabinets in locked rooms, i.e., access is limited to study staff. All study data collection, process, and administrative forms and other reports will be identified by a coded number to maintain participant confidentiality. All records that contain names or other personal identifiers, such as locator or informed consent forms, will be stored separately from study records identified by a code number. All databases will be secured with password-protected access systems. Forms, lists, logbooks, appointment books, and any other listings that link participant ID numbers to other identifying information will be stored in a separate, locked file in an area with limited access.*

Voluntary Nature of the Study. Participation in this study is strictly voluntary, and if agreement to participation is given, it can be withdrawn at any time without prejudice.

Points of Contact. It is understood that should any questions or comments arise regarding this project, or a research related injury is received, the Principal Investigator, *Bauyrzhan Toktarbay, +77021847063, Bauyrzhan.toktarbay@nu.edu.kz* should be contacted. Any other questions or concerns may be addressed to the Nazarbayev University Institutional Research Ethics Committee, resethics@nu.edu.kz.

Statement of Consent.
I_____
_____,
Give my voluntary consent to participate in this study.
The researchers clearly explained to me the background information and objectives of the study and what my participation in this study involves.

I understand that my participation in this study is voluntary. I can at any time and without giving any reasons withdraw my consent, and this will not have any negative consequences for myself.

I understand that the information collected during this study will be treated confidentially.

Signature: _____ Date: _____

Researcher:

Signed_____ Date_____