The enigma of the systemic effects of regular exercise: VO2 max or molecular adaptation pathways

Abstract of PhD Thesis

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“The soul is opened, through the body by the sport”

Albert Szent-Györgyi

1. Introduction and Literature Review

Aging does not affect all living beings equally, as lifespan is influenced by various factors such as body size, lifestyle, and environmental impacts. Human lifespan has also varied significantly over the millennia, becoming longer with the development of civilization and advances in healthcare. The average lifespan has increased, which has brought about new health challenges. The aim of our research was to novelly explore human aging, with a particular focus on the role of physical activity, to contribute to a longer and healthier life expectancy.

1.1. Theories of Aging

According to Gunnar's definition, aging (after a certain period) is the gradual deterioration of the body's functions, leading to a decline in the abilities necessary for survival and reproduction. It is important to distinguish aging from age-related diseases. Aging is a complex process that affects the body on multiple levels, and various theories attempt to explain it. These theories examine genetic, cellular, metabolic, and hormonal changes as well.
Our understanding of aging is continually evolving, as new research can bring forth new theories. Understanding aging is a complex task that still contains many unanswered questions.

1.2. Systemic Effects of Regular Exercise

Exercise has a significant impact on the body. The cardiovascular system can perform better, breathing can become more efficient, and muscles can become stronger. Digestion, the endocrine system, and the nervous system can also improve. The immune system can be strengthened, which can help in fighting diseases. Overall, regular exercise improves the functioning of many organ systems and contributes to health, i.e., it has a systemic positive effect.

1.3. The First Two Generations of Epigenetic Clocks, Classical Epigenetic Clocks

DNA methylation is an epigenetic process in which methyl groups attach to DNA, thereby influencing gene expression. This process can play a key role in human development, aging, and the development of diseases. Epigenetic clocks are able to estimate chronological and biological age by analyzing DNA methylation patterns. The first-generation clocks were Horvath and Hannum's clocks, which estimated chronological age. They are able to estimate calendar age with similar accuracy at different ages and in different samples. Second-generation clocks were able to estimate biological age, which were created by taking into account lifestyle and health factors in addition to methylation patterns. Horvath's colleagues Levin and Lu created the PhenoAge and GrimAge clocks. Thanks to these, it was possible to draw conclusions about the accelerated or slowed aging experienced in biological age and the likelihood of developing diseases.

1.4. The Relationship Between Irisin and Exercise

Irisin is a hormone that is predominantly produced by muscles during physical activity. This protein may play a role in the use of stored fat, blood sugar regulation, reduction of inflammation, protection of cognitive functions, and the development and preservation of bone density. Due to its effects, it has been called the "exercise hormone," as a good correlation can be shown between a healthy lifestyle and elevated hormone levels.

1.5. Redox Balance as a Fitness Indicator

Redox balance, or the balance between free radicals and antioxidants, is essential for health. Regular exercise increases redox capacity, thus providing protection against harmful free radicals. A trained organism can more easily handle the oxidative stress that is harmful to health. With aging, this balance can be disrupted, but regular exercise can help maintain it. Healthy redox balance may be one of the keys to a long and healthy life.

2. Objective

There are several ways to study biological aging. Our research group has focused on epigenetic clocks based on DNA methylation patterns. Based on the scientific results available to us so far, it can be said that the systemic effects of everyday exercise, i.e.,

regular physical activity, include a longer life spent in good health, which is why it seems justified to introduce a test method into the world of epigenetic clocks that examines fitness as well. Our goal is to carry out this research to fill this gap and to shed further light on the relationship between exercise-induced physiological changes and aging. To achieve our goals, we have formulated the following hypotheses: H1 - A new epigenetic clock can be created using partially new methylation sites that takes into account physical fitness. H2 - The new fitness biomarkers can function as independent predictors of health status. H3 - The new epigenetic clock shows a stronger correlation with fitness parameters than the previously existing DNAmGrimAgeAccel. and DNAmPhenoAgeAccel. H4 - Using the new DNAm biomarkers, it is possible to distinguish between the fitness of the samples.

3. Methods

3.1. Development of DNAmFitAge

To investigate the epigenetic effects of lifelong exercise, we first created a DNA methylation-based epigenetic clock that also takes into account fitness metrics, as the existing epigenetic clocks did not consider fitness parameters until the beginning of our study. For this purpose, we developed DNAm biomarkers from four fitness metrics. The fitness metrics were gait speed, maximum handgrip strength, forced expiratory volume in one second (FEV1), and maximal oxygen consumption (VO2 max). Data from three studies were used to develop new DNAm biomarkers that also take into account fitness: Framingham Heart Study Offspring (FHS, n = 1830), Baltimore Longitudinal Study on Aging (BLSA, n = 820), and our rowing study conducted at Lake Velence (n = 307). The FHS study processes the results of longitudinal research on cardiovascular diseases. The BLS study examined the aging of healthy adults. To use the results of different studies simultaneously, we used the method of Key et al., briefly re-centralized and multiplied by the ratio of standard deviation values for each fitness parameter, thus obtaining the same mean and standard deviation. Our validation studies were performed on the results of six additional studies: two Lothian Birth Cohort studies (LBC1921, n = 692; LBC1936, n = 2797), Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE, n = 578), InChianti (n = 924), Jackson Heart Study (JHS, n = 1746)

and Women's Health Initiative (WHI, n = 2117). The new fitness biomarkers were verified on the results of an epigenetic study examining Polish bodybuilders and a control group.

3.2. Evaluation of DNAmFitAge in Bodybuilders

In an independent study, we assessed whether DNAm fitness biomarkers and DNAmFitAge differed significantly between trained male bodybuilders and a control group. A total of 66 male bodybuilders and 149 male control subjects were examined, who had a similar age distribution (p-value > 0.05). Both groups reported how many years they had been training regularly, on average how many intensive workouts they participated in per week, and a total of 88 participants reported what supplements or medications they were taking. The Kruskal-Wallis test was used to examine whether DNAm fitness biomarkers, DNAmFitAge, and FitAge Acceleration differed between the control and trained groups. A linear regression model was used to examine whether the improvement in DNAmFitAge and DNAmVO2max in trained male bodybuilders could be explained by the supplements taken, where DNAmFitAge or DNAmVO2max was the outcome, age as a covariate, and supplementation and fitness status (bodybuilder) as indicator variables. Age was used for correction in the model because age was significantly associated with the use of certain supplements, so if age was not accounted for in the model, the differences observed as a function of DNAmFitAge or DNAmVO2max actually showed differences in the use of supplements. In order for the statistical tests to be appropriate, both the trained and the control groups were defined so that at least 10 people used supplementation. Only six supplements met this criterion: multivitamins (n = 19), protein (n = 17), energy (n = 17) (creatine, pre-workout supplements, energy gels), magnesium (n = 16), vitamin D (n = 14) and omega-3 (n = 12). Furthermore, the Fisher Exact test was used to assess whether these supplements were consumed disproportionately by bodybuilders compared to the control group.

3.3. Analysis of the Rowing Study Sample

In our survey conducted in Hungary, we examined the data of a total of 303 volunteers. All of them completed a consent form for voluntary participation, and the research was approved by the National Institute of Public Health (25167-6/2019/EÜIG). Most of the

participants in the study participated in the World Rowing Masters Regatta held at Lake Velence (n = 203), and the full sample and participation were finalized at the campus of the University of Physical Education and Sports Sciences. The participants in the study were between 33 and 88 years old. During the surveys, they completed a questionnaire that examined their training history, health status, lifestyle, and education. The participants in the rowing world championship formed a heterogeneous sample, as some trained once a week, while others trained every day, so the sample was grouped according to their VO2 max results. Based on the 75th percentile, two groups were defined: a medium-low fitness (MED-LOW FIT) group (men n = 50, women = 62) and a high fitness (HIGH-FIT) group (men n = 93, women n = 91).

3.4. Physiological Tests, Blood Sample Collection

To test short-term memory, a Digit span test was performed. To determine maximum strength, a handgrip dynamometer was used (EH101, place of manufacture: China, year of manufacture: 2017), and a CMJ test was performed using a linear encoder to examine the explosive strength of the lower body (place of manufacture: United States, year of manufacture: 2012). An Omron BF214 scale was used to measure body composition and weight (place of manufacture: Japan, year of manufacture: 2015). To measure maximal oxygen consumption, a step test was performed according to the Chester step test protocol, which estimates relative oxygen capacity based on heart rate values at progressive intensity. Blood was taken from the participants before the VO2 max test. The blood samples were treated depending on which measurement they were used for. Until the tests, the blood samples were stored at -80°C in Eppendorf tubes.

3.5. Irisin Measurement

For irisin analysis, blood was collected in EDTA blood collection tubes, and aprotinin was added to the blood in a 10:1 ratio before storage. The samples were then centrifuged at 1600 g for 15 minutes at 4°C. After centrifugation, the plasma was stored at -80°C. Irisin levels were determined using an ELISA kit (EK-067-29, Irisin Recombination, Phoenix Pharmaceuticals, Inc, Burlingame, USA). Samples from a single subject were always analyzed on the same plate (inter-assay). The coefficient of variation between inter- and intra-assay tests was between 4.1% and 15.2%.

3.6. Determination of Redox Balance

The amount of natural hydroperoxides in the blood was determined spectrophotometrically using the d-ROM test. Concentrations were determined in Carratelli units (UCarr) where 1 UCarr corresponds to 0.8 mg/L hydrogen peroxide. The d-ROMs test was performed with FREE Carpe Diem analysis. To determine antioxidant capacity, a BAP (biological antioxidant power) test was performed. For the test, ferric chloride was mixed with a thiocyanate derivative, which is a special chromogenic substrate. 10 μL of plasma was added to this reaction mixture and incubated at 37°C for 5 minutes. The extent of iron ion reduction was quantified by measuring the absorbance at 505 nm. The BAP test was also performed with FREE Carpe Diem analysis. Redox balance was determined by the BAP/dROM ratio.

3.7. DNA Methylation Measurement

The Infinium MethylationEPIC BeadChip procedure was used to measure DNA methylation (Illumina Inc., San Diego, CA). During the procedure, the EZ-96 DNA Methylation MagPrep Kit was used, which uses a 500 ng DNA bisulfite conversion procedure. The samples were randomized to the test plates. During bisulfite conversion, the DNA binds to 15 μL MagBinding Beads. During the conversion process, the incubation cycles were performed according to the following protocol: 16 cycles of 95°C for 30 seconds, followed by a 1-hour 50°C cycle. After the binding process, the DNA was incubated at 4°C for an additional 10 minutes. In the next step (according to the Illumina Inc., San Diego, CA protocol), the DNA samples were hybridized according to the Illumina MethylationEPIC BeadChip procedure, where 8 μL of bisulfite-treated DNA was the starting material. For quality control of DNA methylation data, minfi, Meffil and ewastool packages were used in the R (version 4.0.0) programming language. Samples that did not meet the criteria defined by Illumina were excluded. The "noob" normalization methodology was used in the R programming language to determine methylation levels. Horvath's online age calculator was used to detail DNAm data and determine aging and the rate of aging (https://dnamage.genetics.ucla.edu/).

3.8. Statistical Analyses

To determine the relationship between the target and predictor variables, multiple linear regression was used while controlling for age and sex. Statistica 13 software was used for statistical analyzes. In the irisin analysis, the possible batch effect was controlled by giving the plates a separate variable in the statistical analysis. The difference between the fitness groups was examined using a two-way ANOVA test, where sex and fitness group were treated as separate test factors; group means were compared using the Tukey HSD test. If the data did not follow the normal distribution evaluated by the Shapiro-Wilk test, the Kruskal-Wallis test was used instead. The relationship between verbal short-term memory and biochemical/physiological markers was analyzed using Spearman rho and Kendall tau calculations. A two-sample t-test and non-parametric Kruskal-Wallis test were used to determine if there was a significant difference between the high-fit and low-med-fit groups in terms of DNAm biomarkers. To eliminate the effect of age observed between the groups, age-corrected DNAm variables were used (FitAge Acceleration, GrimAge Acceleration and PhenoAge Acceleration). T-tests and Kruskal-Wallis tests were performed on fitness parameters such as grip strength and jump (absolute and relative) to serve as reference values for DNAm-based surrogate biomarkers. VO2 max was excluded from the table because this variable was used to form the groups. In addition, DNAmVO2max had to be excluded because the subjects participating in the study were used to create the DNAmVO2max biomarker, so the differences observed between the groups are the results of creating the biomarker.

4. Results

4.1. Relationship of DNAmFitAge to Everyday Exercise

Our analysis of bodybuilders showed that FitAgeAcceleration, DNAmGaitspeed, DNAmGripmax, and DNAmFEV1, as expected, are associated with regular exercise in individuals with low and moderate fitness. The coefficients indicate the effect of a one-unit increase in a DNAm fitness biomarker on physical activity, taking into account age in individuals of the same age group. Their relationship to DNAmFitAge is also as expected; those with a higher FitAgeAcceleration have an older estimated biological age and this is associated with lower physical activity or physical performance. Similarly,

those with faster DNAmGaitspeed, stronger DNAmGripmax, and higher DNAmFEV1 values are physically more active than their same-aged peers. In summary, physically more active individuals showed "fitter" values in terms of FitAgeAcceleration and DNAm fitness biomarkers.

4.2. Age-Dependent Physiological Functions and Blood Markers in the Rowing Study Sample

In addition to creating a new aging biomarker, we examined the previously mentioned physiological parameters and blood markers in our rowing sample. With aging, all examined physiological parameters showed decreased results, both in the MED-LOW-FIT and HIGH-FIT groups. The rate of aging was lower in the HIGH-FIT group, especially in the older age group, but only the jump height differed according to fitness level. Jump height (which is an indicator of anaerobic performance) was the only parameter whose age-related decline could be mitigated by fitness. LDL levels were found to be constant in men, regardless of age and fitness, while HDL levels were higher in fitter men than in their less fit counterparts. A positive correlation was found between HDL and irisin levels, and there was a significant difference in HDL levels between fitness levels (in both sexes). When evaluating the results of short-term memory, we observed that the best results were produced by thin, young and fit subjects.

4.3. DNAmFitAge and Other DNAm Biomarkers in the Rowing Study Sample

The FittAgeAcceleration biomarker showed a stronger relationship with fitness, BMI and blood serum parameters compared to the GrimAge and PhenoAgeAcceleration biomarkers. Furthermore, the aging rate showed the expected data. A positive FitAgeAcceleration predicts a higher biological age than chronological age, while a negative FitAgeAcceleration predicts a younger (fitter) biological age. Every 1-year increase in FitAgeAcceleration was associated with an average decrease of 0.29 (kg/body weight) in grip strength, 0.12 cm (cm/body height) in jump height, 0.31 in HDL level, 0.28 in redox balance, 0.32 in BMI and 0.17 in blood irisin level. The direction of the relationships was the same as with the previously existing biomarkers, such as GrimAge and PhenoAge, but the correlation was strongest with the newly created FitAge biomarker. Of the three biomarkers mentioned, only FitAgeAcceleration showed a

significant difference between the High-Fit and Medium-Low-Fit groups. In women, FitAgeAcceleration was 1.5 years younger in the HF group than in the MLF group ($p=0.005$), while in men the difference was 2.0 years ($p=0.0007$). GrimAgeAcceleration and PhenoAgeAcceleration also showed a younger biological age in favor of the fitter groups in both men and women, but the difference was not significant in either sex ($0.25<p<0.82$). From these results, we can conclude that highly fit women and men are on average 1.5-2 years younger than their low or moderately fit peers in terms of biological age.

4.4. DNAm Fitness Biomarkers in the Rowing Study Sample

Based on the DNAmGripmax, DNAmGaitSpeed and DNAmFEV1 variables, it was possible to distinguish between the fitness status of the female participants in the rowing sample (High-Fit / Med-Low-Fit), but this was not demonstrable in the male sample. Without taking into account whether chronological age is part of the calculation when creating the DNAm biomarker, similar estimated differences can be shown between male fitness groups: 1.49 and 1.44 DNAmGripmax, 0.1 and 0.09 DNAmGaitspeed, 0.14 and 0.15 DNAmFEV1. We concluded that the reason why no difference was shown in the male sample for the listed DNAm biomarkers was that these fitness variables did not differ significantly in the male sample (relative grip strength $p=0.049$, maximum jump $p=0.07$).

5. Conclusions

Since we were able to prove that DNAmFitAge is able to sense physical activity compared to the previously existing biological age determination clocks, and thus can also play a role in determining the quality of life, i.e., it can function similarly to the previously existing clocks, our first hypothesis was confirmed. Namely:

Partially new methylation sites can be used to create an epigenetic clock that takes into account physical fitness. **TRUE**

The newly created DNAm biomarkers have been shown to have significant correlations with mortality, disease status, high blood pressure, time to death, and type 2 diabetes in

validation databases. In addition, it was possible to determine the percentage chance of improvement (in the case of the examined health variables) as a function of the results of the given fitness biomarkers. If the estimation procedure showed a biological age 10 years older than the chronological age, it estimated the chance of mortality to be twice as high compared to individuals of the same sex and calendar age. Overall, it can be said that better DNAm fitness biomarkers are associated with the chance of a disease-free state. Based on this, our second hypothesis was also confirmed.

The new fitness biomarkers can function as independent predictors of health status. **TRUE**

The results of FitAgeAcceleration were as expected, in that it estimated the aging rate of physically fitter subjects to be slower compared to less fit subjects with the same parameters. Due to the limiting factors that emerged during the study, further investigation of the issue is warranted. When evaluating the test parameters, FitAgeAccel also registered a correlation in the same direction as the GrimAgeAccel and PhenoAgeAccel clocks, but the new biomarker showed a stronger relationship with LDL, HDL, fitness parameters, irisin levels, redox balance and BMI variables. Based on these results, the hypothesis could be confirmed, i.e.:

The new epigenetic clock shows a stronger correlation with fitness parameters than the previously existing DNAmGrimAgeAccel and DNAmPhenoAgeAccel. **TRUE**

Summarizing our results, we can conclude that the new biomarker is not only suitable for examining the rate of aging and health status, but also for distinguishing between samples in terms of physical fitness. By examining the FitAgeAccel results, a significant difference could be demonstrated in the rowing study sample, both in men and women, between the groups separated according to vo2 max. With this result, we were also able to confirm our last assumption.

The new DNAm biomarkers can be used to distinguish between the fitness of the samples. **TRUE**

6. Summary

The aim of our research was to explore the profound effects of regular physical activity on biological aging and other molecular processes related to fitness. To this end, we conducted a detailed examination of the physical condition of healthy adults and dedicated athletes. The studies included the analysis of physiological parameters, biochemical markers, cognitive functions, and DNA methylation. By developing our new DNA methylation biomarker, DNAmFitAge, we have gone beyond existing methods. Our results show that regular exercise is associated with a younger biological age, better memory, and a healthier blood profile. These associations suggest that DNAmFitAge can serve as a biomarker to explore the relationship between physical activity and molecular biological processes. The development of DNAmFitAge has opened a new chapter in epigenetic aging research. Our new biomarker can estimate biological age by taking into account grip strength, gait speed, vital capacity and oxygen uptake capacity. This is a novelty because previous models did not take physical fitness into account. DNAmFitAge offers a new approach to measuring biological age by combining physical fitness and epigenetics. The biomarker relies on the already known DNAmGrimAge, which estimates the risk of death, and newly developed DNAm biomarkers based on fitness parameters. The results show that better fitness biomarker values represent a younger biological age in both men and women. We validated our results in five different databases containing individuals with low and moderate physical activity. The study also found that FitAgeAcceleration, a new epigenetic aging rate biomarker sensitive to physical fitness, is closely associated with age-related diseases and can predict time to death and the onset of cardiovascular diseases. Our results support the idea that regular exercise can slow down the aging process and improve health. Everyday exercise has an extremely complex effect on our body, which is undoubtedly underpinned by molecular-level adaptations. One of the most important effects is the increase in oxygen utilization capacity, which contributes to the more efficient functioning of many organ systems. Higher than average physical fitness is closely associated with a longer lifespan. The aim of our research is to gain a deeper understanding of the complex relationship between aging, fitness and the underlying biochemical processes. With our studies, we open up new perspectives in understanding the functioning of the human body, with particular emphasis on epigenetic and aging processes. The new biomarkers we have created may

be useful in the future for determining fitness based on DNA methylation, and even for developing exercise intervention programs, such as an irisin level-increasing therapy (exercise-induced irisin level increase), which may also slow down the aging process.

7. List of Own Publications

List of publications related to the dissertation:

Jokai M, Torma F, McGreevy KM, Koltai E, Bori Z, Babszki G, Bakonyi P, Gombos Z, Gyorgy B, Aczel D, Toth L, Osvath P, Fridvalszky M, Teglas T, Posa A, Kujach S, Olek R, Kawamura T, Seki Y, Suzuki K, Tanisawa K, Goto S, Kerepesi C, Boldogh I, Ba X, Davies KJA, Horvath S, Radak Z. (2023) DNA methylation clock DNAmFitAge shows regular exercise is associated with slower aging and systemic adaptation. Geroscience, 5:2805-2817.

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List of publications independent of the dissertation:

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Kawamura T, Radak Z, Tabata H, Akiyama H, Nakamura N, Kawakami R, Ito T, Usui C, Jokai M, Torma F, Kim HK, Miyachi M, Torii S, Suzuki K, Ishii K, Sakamoto S, Oka K, Higuchi M, Muraoka I, McGreevy KM, Horvath S, Tanisawa K. (2024) Associations between cardiorespiratory fitness and lifestyle-related factors with DNA methylation-based ageing clocks in older men: WASEDA'S Health Study.Aging Cell, 1:e13960.

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Torma F, Bakonyi P, Regdon Z, Gombos Z, Jokai M, Babszki G, Fridvalszki M, Virág L, Naito H, Iftikhar Bukhari SR, Radak Z. (2021) Blood flow restriction during the resting periods of high-intensity resistance training does not alter performance but decreases MIR-1 and MIR-133A levels in human skeletal muscle.Sports Med Health Sci, 1:40-45.

Seki Y, Aczel D, Torma F, Jokai M, Boros A, Suzuki K, Higuchi M, Tanisawa K, Boldogh I, Horvath S, Radak Z. (2023) No strong association among epigenetic modifications by DNA methylation, telomere length, and physical fitness in biological aging.Biogerontology, 2:245-255.

Aczel D, Torma F, Jokai M, McGreevy K, Boros A, Seki Y, Boldogh I, Horvath S, Radak Z. (2023) The Circulating Level of Klotho Is Not Dependent upon Physical Fitness and Age-Associated Methylation Increases at the Promoter Region of the Klotho Gene.Genes (Basel), 2.