Examination of the epigenetic changes that occur as a result of several decades of exercise

Abstract of PhD Thesis

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1. Introduction

Aging

Aging is a natural and automatic biological process, an inevitable consequence of life. The aging body is characterized by a gradual decline in the functions of tissues and organs, a worsening quality of life, and an increase in the risk of death. Nowadays, thanks to better living conditions and improved health care, the expected average age at birth is being pushed forward dramatically, and thus the proportion of the elderly in the population is increasing. Aging, in addition to being universal, has significant unique characteristics. In addition to genetic factors, differences in lifestyle can contribute significantly to individual differences. During our lives, our body is subjected to various external and internal influences, which can accumulate in the form of mutations and leave traces in our DNA. Aging affects gene expression in such a way that when the aging of the genetic material reaches a certain threshold, gene expression ceases. Overall, these damages increasingly hinder healthy functioning, and can lead to the development of tumors, cardiovascular and neurodegenerative diseases, and ultimately to death.The question therefore arises whether the process can be influenced through a more in-depth knowledge of aging. Recent research suggests that aging can be slowed or even reversed by altering gene activity. However, for this, we need to know more precisely the key players in the aging process. Studies on human models have shown that mainly genetic differences and somatic mutations acquired during our lives can be behind the different aging processes. In addition to all this, we can attribute an important role to "non-genetic factors", thus supporting the importance of "epi"-genetic mechanisms in the regulation of longevity. Such "factors" are, for example, calorie deprivation, a reduction in the rate of basic metabolism, an increased oxidative stress response, and the restoration of the balance of mitochondrial proteins. The factors mentioned above can all be related to the extension of life.

Signaling of aging

The signaling of aging, if we take it that way, is also that of longevity. Genes related to the pathway are also called aging or longevity genes. If the efficient operation of the road is preserved or defects are eliminated in time, aging can be delayed, if not completely avoided. However, for this, we need to know more about the signaling of aging and its changes with age. The joint, coordinated, and preserved operation of the signal transmission paths connected to the road is necessary for a long life. Based on the "incoming signals", we can conclude that

aging can be affected by 1) different nutritional characteristics, 2) certain hormones, and 3) oxidative stress.

Hormones in the signaling of aging, KL: Among the hormones that play a role in aging, insulin, and growth hormone play a particularly important role. However, a third hormone also affects the signaling of aging, and this is Klotho. Protein is considered a significant biomarker of longevity. The KL gene is located on chromosome 13 in humans. The promoter region of the gene is extremely rich in CpG islands. The methylation status of the promoter region is likely related to the expression of KL mRNA. The extracellular domain of tKL is cleaved by membrane-bound proteases. First, soluble KL is produced, and then after a signal peptide is placed on it, secreted KL is produced, which is secreted into the blood, urine, and cerebrospinal fluid. sKL functions as a circulating hormone that regulates many receptors and ion channels on the cell surface, has a beneficial effect on inflammatory processes, and participates in protection against oxidative stress. So the question arises, can keeping the level of KL constant, or replenishing the protein, promote healthier aging and have a positive effect on the prevention of diseases?

Oxidative stress signaling pathway: During biological oxidation, the ingested nutrient is transformed into usable energy for the cell, i.e. ATP, the terminal step of which takes place in the mitochondria. These organelles play a central role in aging. If the oxidation reactions taking place in the mitochondria suffer damage (see aging), not only energy production decreases, but reactive oxygen derivatives (ROS) increase as a byproduct of the process. The released free radicals are eliminated by the enzymes of the redox household, the proper functioning of which is therefore essential for healthy aging and thus longevity. If the balance of the household is upset, the excess free radicals, due to their natural affinity, spontaneously react with certain molecules, including DNA bases.

The role of exercise over decades in aging

The extremely important role of regular physical activity in maintaining good health and a high quality of life for as long as possible has been known for a long time. With the advent of cars and public transportation, the simplification of communication, and the sedentary and overburdened, stressful lifestyle, recreational trends, and leisure-time exercise are becoming increasingly important. We know that sport not only improves physical performance but also lays the foundation for healthy physical, mental, and spiritual functioning. In addition to the decline in physical performance experienced during aging, there is also a decline in motor and

cognitive functions. These dysfunctions represent a heavy burden, both at the level of the individual and at the level of families and society in general. It is known that regular physical activity counteracts the negative effects associated with aging, reduces mortality and morbidity, and extends an active lifespan. Regarding the relationship between aging and exercise, we know, for example, that the value of maximal oxygen uptake (VO2max) over the age of 50 shows a negative correlation with mortality. Regular physical exercise is effective, for example, against the deterioration of heart and blood vessel function associated with increasing age, which are among the leading causes of death in Hungary. Physical exercise effectively combats osteoporosis in old age, improves coordination, and helps maintain muscle mass, which is useful for avoiding falls and accidents. In addition, in connection with age, the skeletal muscle accumulates more and more oxidative damage and free radicals, which regular exercise can counteract by increasing the antioxidant capacity.

"The Aging Telomeres":

One of the main causes of aging is the critical shortening of telomere lengths. Telomeres are repetitive, TTAGGG-rich, non-coding nucleotide stretches at the ends of chromosomes, typically between 3 and 20 kilobases in length in humans. Their job is to protect the DNA from the shortening that occurs every cell cycle. As long as these approximately 100 base pair deletions only affect the telomere region that does not contain genes, the coding section of the DNA is not damaged. However, after a certain number of cell divisions, when the shortening of the telomere length is already critical, the dividing cells can no longer duplicate, thus reaching the state of cellular senescence. This means roughly 50 cell divisions in human cells. The longer the telomere section, the more the cell can divide without damaging its gene pool. Although the enzyme telomerase can add de novo base pairs to telomere stretches, telomeres shorten with age due to continuous cell division. It is an interesting fact that significant oxidative stress in the aging process, for example, accelerates the shortening of telomeres. There is a strong link between telomere length and age-related diseases. It has been described by their length inversely correlates with the risk of many age-related diseases, such as atherosclerosis, stroke, obesity, and myocardial infarction. The "wear and tear" of telomeres shows a positive correlation with diabetes and the development of related complications. Furthermore, several studies have shown reduced leukocyte telomere length in tumors, such as colon tumors.

The effect of regular exercise on telomere length

We know that exercise has a beneficial effect on the length of telomeres. Even a 12-week, low-frequency, moderate-intensity, short-term training program had a positive effect on telomere length and was positively correlated with redox homeostasis. Movement presumably affects telomere length either by influencing the activity of telomerase, regulating oxidative stress, initiating/inhibiting inflammatory processes, or influencing the content of skeletal muscle satellite cells. According to some studies, more regular or more intense physical activity is associated with longer telomere length. This difference is more pronounced in the case of the elderly, which may indicate that physical activity has a positive effect on the age-related decrease in telomere length. Relatedly, the telomere length of leukocytes and skeletal muscle cells is positively related to a healthy lifestyle. On the other hand, however, there are quite a few publications that do not describe differences between athletes and non-athletes in terms of telomere lengths. Thus, the relationship between physical activity and telomere lengths remains controversial.

Epigenetics

Although we have known for a long time that gene function can be modified not only by changes in the sequence of genes, attention has mostly turned to epigenetics only in the last twenty years. Waddington already introduced the concept of "epigenetics" in 1942. In the word, the ancient Greek prefix "epi" meaning "above" refers to the transfer of genetic information without changing the DNA sequence. The scope of epigenetics therefore includes mitotically and/or meiotically inherited processes, environmental effects, and DNA changes due to nutrition, exercise, and aging, which lead to changes in the expression of the genes that determine the phenotype in such a way that the sequence of DNA nucleotides is not modification is made. Epigenetic processes are natural and essential for the proper functioning of the body. There is a problem when these processes do not take place properly and thus cause serious health and behavioral disorders. Unlike genetic changes, epigenetic processes are reversible. Although we inherited a significant part of the methylation patterns from our parents, they can be affected by various external and internal factors. During its development, the body is affected by many external stimuli, such as the food consumed, physical activity, and stress. In addition, epigenetic processes can be influenced by various environmental agents: heavy metals, pesticides, exhaust gasses, tobacco smoke, polycyclic aromatic hydrocarbons, radioactivity, viruses, and bacteria. Furthermore, even aging itself is such an internal factor. Global hypomethylation with aging causes genetic instability and spontaneous mutations. Nowadays, it is already known that epigenetic changes are behind most diseases, including

cancer, cognitive dysfunctions, and various respiratory, cardiovascular, reproductive, and autoimmune diseases.

DNA methylation

One of the most significant modifications to DNA, and therefore one of the most frequently researched, is DNA methylation. Its popularity is because it can be studied very easily with existing technologies: bisulfite conversion coupled to PCR, pyrosequencing, microarrays, and deep sequencing can all be suitable for examining DNA methylation. Due to the simplicity of sample preparation and the cost-effective techniques, it is also possible to carry out large-scale tests. DNA methylation refers to the modification of nucleotides, the "building blocks" of DNA, with a methyl group. In humans, during methylation, the cytosine bases that can bind to guanine nucleotides through phosphate groups, resulting in so-called CpG dinucleotides, play a particularly important role. These CpG dinucleotide units are unevenly distributed in the genome, most of them occur in the area of so-called CpG islands. CpG islands are typically found in large numbers in the promoter and regulatory regions of various genes. In healthy body cells of human tissues, more than 70-80% of CpG dinucleotides are in the methylated state, which represents almost 1% of the entire genome. The most typical methylated base in the genome of eukaryotes is 5-methylcytosine (5mc), which means that a methyl group is placed on the fifth carbon atom of cytosine. Methylation changes can lead to the enhancement of gene expression or the silencing of a given gene. The applied methyl groups physically inhibit the binding of TF to the DNA double helix. It follows that, while TFs arriving in the cell nucleus at low or completely unmethylated DNA promoter sections can start gene transcription unhindered, in highly DNA-methylated regions, the binding of TFs to the DNA strand becomes impossible, which typically results in the inhibition of transcription.

The epigenetic clocks

Research in recent years has revealed that the biological age of tissues can be determined based on certain DNA methylation biomarkers. Epigenetic age is closely related to chronological aging but also takes environmental and lifestyle effects into account. The so-called second-generation clocks, such as DNAmPhenoAge and DNAmGrimAge. These are more sensitive markers of mortality risk and have also been shown to be more accurate in predicting many age-related diseases:

- PhenoAge is a measure of biological age that was created based on nine clinical characteristics and chronological age. This "phenotypic age" was predicted based on the methylation status of 513 CpGs by Levine et al. Based on the clock, it can be estimated whether the examined individual is younger or older than their chronological age (DNAmPhenoAge acceleration/AgeAccelPheno), i.e. their aging is accelerated (negative sign) or slowed down (positive sign), as well as the chance of death in the next 10 years.
- In the case of creating DNAmGrimAge, with the difference that they took into account the methylation of plasma proteins associated with morbidity or mortality and smoking. "GrimAge acceleration" (DNAmGrimAge acceleration/AgeAccelGrim), similar to DNAmPhenoAge acceleration, is the "residual" of the chronological age-adjusted regression, i.e. the difference between chronological and Grim age.
- DNA methylation-based estimation of telomere length, DNAmTL, also outperforms measured telomere length in predicting time to death and age-related diseases.

2. Objectives

We set out to examine:

1. What is the effect of sport on the methylation of the promoter region of the KL gene?
2. What is the effect of training on the level of "protein of youth" in master athletes? Does the sKL protein level show a correlation with anthropometric, exercise physiology, and cognitive parameters?
3. Is there a relationship between circulating sKL and epigenetic clocks based on DNA methylation in master athletes?
4. What is the relationship between physiological test results and RT-PCR measured (TL) and DNA methylation-based TL (DNAmTL) values?
5. Is there a relationship between measured and estimated telomere lengths and epigenetic clocks?

We formulated our hypotheses as follows.

1. Examining the "protein of youth", we assumed that:

1.1. There is a connection between the level of "protein of youth" in senior athletes and the redox balance of the body.

1.2. Decades of exercise modify the methylation of the promoter region of the KL gene and play a role in the relationship between sKL and physical fitness.

1.3. There is a relationship between the level of KL and epigenetic clocks, and thus the level of the hormone can influence the speed of epigenetic aging.

2. Examining the telomere length of senior athletes, we assumed that:

2.1. Decades of exercise causes changes in measured (TL) and estimated (DNAmTL) telomere length.

2.2. The level of physical strength/fitness affects telomere lengths.

2.3. The relationship between telomere lengths and epigenetic clocks is modified in master athletes compared to the control group.

3. Material and method

3.1. Persons participating in research

The study was approved by the National Center for Public Health, by the Declaration of Helsinki, and the regulations in force in Hungary, our TUKEB ethics license number 25167-6/2019/EÜIG. For our study, the sampling took place at the Masters World Rowing Championships in Venice in 2019. We took a sample from the 194 senior competitors, which was followed by the sampling of a control group consisting of 109 people in Budapest. A total of 303 people participated in the tests. The age of the participants was between 37 and 85 years. Participation in the research was voluntary, the participants filled out a written consent form. All participants filled out a questionnaire regarding their health status and lifestyle, dietary habits, and of course their exercise habits. The senior rowing group proved to be very heterogeneous; many athletes only had one or two training sessions per week, while others did training work daily. Among the participants, 267 people followed a normal diet, 19 people were vegetarians and 17 people followed other types of diet (gluten-free, paleo, lactose-free, DM diet). The middle-aged and elderly people participating in our study were classified into master sportsmen (MF), master sportswomen (EN), and controls (control men (KF), control women (KN).

3.2. Anthropometric, exercise physiology, and cognitive tests

Our sample collection was preceded by a series of multi-stage tests. During the anthropometric examination, body weight and body height were measured, and the body mass index of the subjects was also determined. The exercise physiology test series consisted of four tests; a maximum hand grip strength measurement, a maximum jump measurement, a Chester-step test, and a cognitive test.

3.3. Blood sampling, hematology, and biochemical tests

Blood was collected from the subject's elbow vein into blood collection tubes containing K2-EDTA, ACDA anticoagulant, and CAT (Serum Sep Clot Activator Tubes) serum, after the anthropometric, exercise physiology and cognitive tests, before the Chester-step test. The tubes containing the EDTA anticoagulant and the CAT serum were cooled, transported from the site within 2 hours, and processed in our laboratory. The blood collection tubes containing the ACDA anticoagulant were grouted on site and kept on dry ice at the end of the day in our laboratory's -70-degree cooler. The blood samples treated with K2-EDTA anticoagulant and ACDA anticoagulant were centrifuged at a speed of 1600g for 15 minutes at 4 degrees Celsius (Sigma 1-16K (Refrigerated Microfuge) Osterode am Harz, Germany), then the blood plasma component was pipetted into Eppendorf tubes. The plasma samples obtained in this way, as well as the remaining cellular elements, were stored at -70 degrees Celsius. The contents of the CAT serum blood collection tubes were divided into two parts after centrifugation (3000 rpm, 10 minutes, room temperature, SERVOSpin Plus centrifuge, Germany). From one part, 8 blood chemistry parameters (glucose, cholesterol, LDL, HDL, triglyceride, liver enzymes (GOT, GPT, gamma GT)) were determined.

3.4. DNA isolation

Afterward, DNA was isolated in our laboratory from the cellular elements of the samples treated with the K2-EDTA anticoagulant, including the "buffy coat" containing white blood cells, using a DNA isolation kit (Pure LinkTM Genomic DNA Mini Kit, Thermo Fisher, Carlsbad, CA, USA). The kit was applied according to the manufacturer's instructions.

3.5. Methylation studies

The first step in the methylation assay is a bisulfite conversion. The essence of the process is that the methylated cytosine bases "remain unchanged" due to the presence of the methyl group. While non-methylated cytosine nucleotides are converted to uracil and later to thymine as a result of bisulfite conversion. Depending on whether the cytosine to be tested is methylated or unmethylated, there is a difference in the sequence of DNA nucleotides. As a starting point for the procedure, 500 ng of genomic DNA is converted with hydrogen sulfite, for which an EZ-96 DNA methylation MagPrep Kit (Zymo Research, Irvine, CA, USA) and a KingFisher Flex robot (Thermo Fisher Scientific, Breda, The Netherlands) are used. we used The samples were laid out in a random order. Bisulfite conversion was performed according to the manufacturer's

protocol, with the following modifications: 15 µl MagBinding Beads were used to bind the DNA. The conversion reagent was incubated according to the following cycle protocol: 16 cycles at 95 °C for 30 seconds, then at 50 °C for 1 hour. After the cycle, the DNA was incubated for ten minutes at 4 °C. Product DNA samples were then hybridized to an 850K Infinium MethylationEPIC BeadChip (Illumina Inc., San Diego, CA) according to the manufacturer's protocol with the modification that 8 µl of bisulfite-treated DNA was used as starting material. This chip can determine the methylation of CpG islands at more than 850,000 points. Essentially, the methylation status of the given cytosine can be determined with the help of primers capable of binding to methylated or unmethylated DNA marked with different colored fluorophores. Quality control of DNA methylation data was performed with Meffil and Ewastools packages, R version 4.0.0. Samples that did not meet the quality criteria set by Illumina (including extension, hybridization, and bisulfite conversion) were excluded from the study. To determine the methylation level, we used "Noob" normalization in R. Horvath's online age calculator was used to process the methylation data and calculate the rate of aging.

3.6. Determination of the plasma level of sKL protein

Plasma levels of sKL were determined from samples treated with ACDA anticoagulant. The blood samples were centrifuged at a speed of 6000xg for 15 minutes at 4 degrees Celsius (Sigma 1-16K (Refrigerated Microfuge) Osterode am Harz, Germany), then the blood plasma component was separated with a pipette and stored at -80 degrees Celsius until the measurement. Samples and reagents were prepared as described in the protocol. To measure the protein level, we used an enzyme-linked immunosorbent assay (ELISA), including the Human Klotho ELISA kit, according to the manufacturer's instructions (R&D Systems, DuoSet ELISA, Cat #DY5334-05, Minneapolis, MN, USA). The measurements were performed on a 96-well microplate, on which the standard row was measured in duplicate, as well as the samples in one copy. The test standard curve range was 70-7000 pg/ml. The optical density was read with an ELISA reader (Thermo Labsystems Multiskan EX, Vantaa, Finland) at 450 nm and 595 nm, and after subtracting the background (595 nm), the amount of sKL was calculated in pg/ml. The natural logarithm (ln) of the values thus obtained was taken for further analyses.

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3.7. Telomere length determination

In our study, we determined the length of telomeres using two methods:

3.7.1. RT-PCR-based telomere length determination

The mean telomere length (TL) of genomic DNA samples from whole blood was determined by Cawthon's PCR-based method using a commercially available PCR kit (ScienCell Research Laboratories Inc., San Diego CA Catalog no. #8908). The kit was used according to the manufacturer's recommendations. During the PCR reaction, telomere-specific primers recognize and amplify telomere sequences. For each DNA sample, two consecutive reactions were performed. The first reaction was used to amplify a single-copy reference (SCR) gene. The primer pair used here recognizes and amplifies a 100 bp-long region on human chromosome 17 and serves as a reference when calculating the telomere length of the target samples. The second reaction was aimed at the telomere sequence. PCR reactions were performed in a final volume of 20 µl, 5 ng reference/genomic DNA sample (final concentration = 0.625 ng/µl), 1-1 µl telomeric primer and 10 µl 2x Master Mix were used. PCR reactions were performed on a PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) in the following environment: first 95 °C for 10 min, then 32 cycles of 95 °C for 20 s, 52 °C for 20 seconds, and finally 72 °C for 45 seconds. In each case, three parallel measurements were performed on the same sample.

3.7.2. Estimation of telomere length

In addition to the measurement, the length of the telomeres can also be estimated. For this, software developed by Lu et al. was available to estimate methylation-based telomere length (DNAmTL).

3.8. Determination of redox homeostasis

The amount of hydrogen peroxide (H_2O_2) in the blood was determined using the spectrophotometric method, using the d-Roms (derivatives of reactive oxygen metabolites) test, as previously described. We used a FREE Carpe Diem analyzer (Wismerll Co., Ltd., Tokyo, Japan) for the d-Roms test. The concentrations determined in this way were expressed in traditional units (Carratelli units; UCarr), where 1 UCarr corresponds to 0.8 mg/l H_2O_2. The iron-reducing capacity of the plasma, i.e. the redox homeostasis, was estimated using the biological antioxidant power (BAP) test. In this test, ferric chloride is mixed with a special chromogenic substrate, a thiocyanate derivative, to blood plasma samples (10 µl) and incubated at 37 °C for 5 minutes. The reduction of the iron ion can be inferred from the absorbance measured at 505 nm. BAP measurements were also performed with a FREE Carpe Diem analyzer. The redox balance was estimated based on the BAP/dROM ratio (µmmol/l/UCarr).

3.9. Statistical analysis

The results were subjected to statistical tests. The Statistica 13 program (TIBCO) was used during the analyses. First, the normality of the variables was tested with the Shapiro-Wilk test to apply the appropriate parametric and non-parametric tests. In our studies, we used a two-sample t-test to detect the difference between the groups. To detect the relationships, we performed Pearson's correlation, where KL levels and telomere lengths were the dependent variables. The significance level was defined as $p<0.05$.

4. Results

4.1. Examination of the sKL level in master athletes

The sKL protein level was determined in 202 individuals. In the case of men, the average age of the master athlete group and the control group did not differ significantly from each other (MF vs. KF; p=0.5297). In women, however, the control group was significantly older (MN vs. KN; p<0.0001). In our cohort, the serum level of sKL protein showed a decrease with advancing age, that is, it was negatively correlated with age (partial r= -0.14; p=0.0439). There is also a significant negative relationship between the age of participants in the World Rowing Masters Championship and the serum level of sKL (partial r= -0.19; p=0.0295). On the other hand, there was no detectable correlation between sKL and age in the case of the control group (partial r= -0.065; p=0.5925).

Examining the difference between the sexes, the male gender was associated with significantly higher sKL values (male: 6.05±0.12 pg/ml; female: 5.55±0.11 pg/ml; p=0.002*). There were no significant differences in sKL levels between the master athlete and control groups in either gender (male master athlete: 5.92±0.09 pg/ml; male control: 6.13±0.23 pg/ml; p =0.4080; female master athlete: 5.39±0.15 pg/ml; female control: 5.71±0.15 pg/ml; p=0.1459.

The anthropometric, exercise physiology, and cognitive data showed a correlation with the sKL level as follows: higher maximum hand grip strength was associated with a higher sKL level when examining the master sports group (partial r= 0.24; p=0.0058). On the other hand, the maximum hand grip strength of the control group showed no correlation with the sKL level (partial r= 0.19; p=0.1142). There is no relationship between the results of the cognitive test, the results of the vertical jump test, and the VO2max values estimated with the Chester-step test and the sKL levels, in the case of either group.

Examining the relationship between sugar management (random blood sugar level) and sKL, we found no significant relationship, only a negative trend in the master athlete group (partial r= -0.15; p= 0.0900).

Examining the correlation between the sKL level and DNAmPhenoAge and DNAmGrimAge, the data showed that sKL is related to PhenoAge and that higher sKL level was associated with slower DNAmPhenoAge acceleration in the master athlete group (partial r= -0.21; p=0.0192). However, this correlation did not exist in the case of the control group (partial r= -0.17; p=0.1587). Examining the relationship between DNAmGrimAge and sKL, a negative trend can be seen in the master athlete group (master athlete group: partial r= -0.16; p=0.0740; control group: partial r= -0.06; p=0.6370). However, the acceleration of DNAmGrimAge did not show

any relationship with the level of sKL, in the case of either group (master athlete group: partial r= 0.02; p=0.8133; control group: r= 0.02; p=0.8580).

The redox balance we measured and the level of sKL showed a significant positive correlation when examining the master athlete group (partial r=0.28; p=0.0115). However, in the case of the control group, we did not find a significant relationship between redox balance and sKL level (r=0.17; p=0.1497). In master athletes, the higher the H_2O_2 level, the lower the sKL level (partial r= -0.25; p=0.0242), but the correlation was not shown in the control group (partial r= -0.21; p=0,0802).

The methylation level (beta value) of 13 CpGs in the promoter region of the KL gene was determined. Based on these, we found that the age-related decrease in sKL level was associated with higher methylation of the promoter region of the KL gene. In the case of men, after correcting for age, we found a different region-specific methylation pattern between the master athlete and the control group, which was not the case for women.

4.2. Examination of the measured and estimated telomere length of senior athletes

Next, we examined the measured (TL) and DNA methylation-estimated (DNAmTL) values of the senior athletes and the control group. The average telomere lengths measured in the whole blood genome were compared in 241 people.

Looking at the whole cohort, the measured telomere lengths (TL) showed a negative correlation with age (partial r= -0.23 p=0.0003). However, the relationship between TL and age was no longer detectable in the case of senior athletes (partial r= -0.03; p=0.7142), while the negative correlation still existed in the control group (partial r= -0.35; p=0.0004). In our study, we also found a strong, negative correlation between estimated telomere length (DNAmTL) and age (partial r= -0.75; p<0.0001). This relationship remained for each group, separately (DNAmTL senior athletes: partial r= -0.74; p< 0.0001; Fig. 12C; DNAmTL control: partial r= -0.77; p< 0 .0001).

In terms of gender, the correlation between measured and estimated telomere lengths and age in both cases was stronger for women: TL women: partial r= -0.73; p<0.05; TL men: partial r= -0.66; p<0.05; and DNAmTL women: partial r= -0.78; p<0.0001; DNAmTL men: partial r= -0.76; p<0.0001. The averages of the measured telomere lengths of men and women were the same (TL women: 8.56; TL men: 8.57; p=0.8168), while the estimated telomere lengths were

significantly longer in the case of women (DNAmTL women: 6.9; DNAmTL men: 6.7; p=0.0002).

In the case of men and women, the following differences emerged between the senior athletes and the control groups: in master athletes, a longer telomere length was measured in almost all cases for both sexes. Exception: the measured telomere lengths were longer in the female control group.

We showed the following correlations between the anthropometric, exercise physiology, and cognitive data and the DNAmTL: we saw a positive relationship between the estimated telomere lengths and the maximum jumping height in both the master and control groups. The grip force/body weight ratio and the result of the cognitive test in the control group showed a positive relationship with the telomere length, which was not the case in the master group. There was no detectable relationship between telomere length and body mass index.

The maximum oxygen uptake capacity, i.e. VO2max was measured neither by the master athlete (partial r= -0.1; p=0.2286) nor by the control group (partial r= 0.12; p=0.2488) He showed no connection with his TLs. However, the estimated VO2max values were positively correlated with DNAmTL in the case of the control group (partial r=0.29; p=0.0067), a positive relationship that no longer existed in the master athlete group (partial r=0.08; p=0.3414).

No relationship could be detected between the measured TLs of master athletes and epigenetic clocks (DNAmPhenoAge: TL master athlete: partial r= -0.11 p=0.2014; DNAmGrimAge: TL master athlete: partial r= -0.12 p= 0.1574). In contrast, in the control group, we found a negative relationship between TLs and markers of epigenetic aging (DNAmPhenoAge: TL control: r= -0.34; p=0.0007; DNAmGrimAge: TL control: partial r = -0 .36, p = .0004). We found a strong relationship between the DNA-based TL estimation and DNAmPhenoAge and DNAmGrimAge, which did not change in master athletes either. The relationship between DNAmPhenoAge acceleration and DNAmTL was not significant in the case of senior athletes. Examining the relationship between the acceleration of DNAmGrimAge and DNAmTL, the results showed that longer telomeres were associated with slowed aging in both master athletes and controls.

The relationship between redox homeostasis and telomere lengths was as follows: The measured TL lengths showed a positive relationship with redox homeostasis in the case of athletes (partial r=0.26; p=0.0106), while there was no relationship in the case of the control

group (partial r=0.17; p=0.0925). The relationship between TL and H2O2 was found to be significantly negative in senior athletes (partial r=-0.23; p=0.0213), while only a positive trend can be seen in non-athletes (partial r=0.20; p=0.0517).

5. Conclusions

During our research, we set ourselves the goal of examining the effect of decades of exercise on the molecular processes of aging. We approached the broad topic of aging from the perspective of examining the "youth gene" and telomere lengths. We investigated the effect of sport on the "gene of youth", including changes in the methylation of the promoter region of the KL gene in master athletes. However, there were differences not only in the methylation of the promoter region but also in the transcribed/translated protein levels that we also measured as a result of decades of training. The anthropometric, exercise physiology, and cognitive parameters can well show the current state of the individual in all respects, which is why we investigated their relationship with sKL. This study showed a correlation between the maximum hand grip strength of senior athletes and the "protein of youth". In the master athlete group, in addition to the decrease in sKL level with age, we saw a negative relationship with the acceleration of DNAmPhenoAge, and in the case of DNAmGrimAge, a trend in the same direction. The correlation shown between these indicators of biological age and the level of sKL makes it possible to insert a new mosaic piece into the "big whole" as far as aging research is concerned. In addition, another marker of aging, telomere length, was examined in master athletes. Based on our results, it can be seen that estimating telomere length based on DNA methylation is a more sensitive method for examining the relationship between telomere length and physical fitness than RT-PCR-based TL length measurement. The decrease in the length of telomeres with advancing age "disappears" under the influence of sport, that is, according to our assumption, sport overrides this. The same effect applies to maximal hand grip strength, VO2max, cognitive test results, and epigenetic clocks, DNAmPhenoAge and DNAmGrimAge.

Examining our hypotheses, we can conclude the following:

1. Examining the "protein of youth", we assumed that:

1.1. There is a connection between the level of "protein of youth" in senior athletes and the redox balance of the body. TRUE

1.2. Decades of exercise modify the methylation of the promoter region of the KL gene and play a role in the relationship between sKL and physical fitness. PARTLY TRUE

1.3. There is a relationship between the level of KL and epigenetic clocks, and thus the level of the hormone can influence the speed of epigenetic aging. PARTLY TRUE

2. Examining the telomere length of senior athletes, we assumed that:

2.1. Decades of exercise causes changes in measured (TL) and estimated (DNAmTL) telomere length. TRUE

2.2. The level of physical strength/fitness affects telomere lengths. TRUE

2.3. The relationship between telomere lengths and epigenetic clocks is modified in master athletes compared to the control group. TRUE

List of own publications

Announcements related to the dissertation

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