UNIVERSITAT DE BARCELONA

Facultat de Farmàcia
i Ciències de l'Alimentació

FACULTAT DE FARMÀCIA

Treball Final de Grau

Grau de Farmàcia

Physical exercise impact on aging-related pathways across generations in *C. elegans*

MARTA RIBALTA VILELLA

Farmacologia i Terapèutica

Bioquímica i Biologia Molecular

Salut Pública

Departament de Farmacologia, Toxicologia i Química Terapèutica

Facultat de Farmàcia i Ciències de l'Alimentació

Universitat de Barcelona

Barcelona, 14 de juny de 2023

Abstract

Aging has been defined as a gradual functional decline with a progressive physiological integrity loss, increasing the organism's vulnerability to death. Otherwise, this described deterioration is the major risk factor for most current human pathologies, including neurodegenerative diseases, cancer, cardiovascular disorders, and diabetes. However, in the last decades, non-pharmacological treatments like physical exercise have provided general health benefits against aging decline. In this study, we aim to analyze how lifestyle factors such as physical exercise can modify the molecular expression of aging-related pathways and observe if this gene expression modification pattern is inherited transgenerationally throughout the following generations.

Among all hallmarks of aging process, in this study we highlight epigenetic alterations. Even though extensive studies of transgenerational epigenetic inheritance have been reported in *Caenorhabditis elegans*, there is still a gap regarding how physical exercise might benefit organisms through epigenetic pathways. Then, we implement a physical exercise treatment in the first *C. elegans* generation, and we analyze the subsequent generations to study transgenerational heritable changes in gene expression involved in aging-related pathways. We emphasized on CREB transcription factor pathway, early growth response 1 (EGR1) transcription factor, superoxide dismutase 1 (Sod1) as a powerful antioxidant, and disintegrin and metalloproteinase domain-containing protein (ADAM10) pathway since its role in correct neurological development.

Key words: exercise, *C. elegans*, aging, transgenerational epigenetic

Resum

L'envelliment s'ha definit com un declivi funcional gradual amb una pèrdua progressiva de l'integritat fisiològica, augmentant la vulnerabilitat de l'organisme a la mort. Altrament, aquest deteriorament descrit és el principal factor de risc per a la majoria de patologies humanes actuals, incloses les malalties neurodegeneratives, el càncer, els trastorns cardiovasculars i la diabetis. No obstant, en les últimes dècades, els tractaments no farmacològics com l'exercici físic han proporcionat beneficis generals per a la salut cara l'envelliment. En aquest estudi, pretenem analitzar com factors de l'estil de vida com l'exercici físic poden modificar l'expressió molecular de vies relacionades amb l'envelliment i observar si aquesta modificació de l'expressió gènica s'hereta transgeneracionalment.

Entre tots els processos distintius de l'envelliment, en aquest estudi destaquem les alteracions epigenètiques. Tot i que estudis extensos s'han realitzat sobre l'herència epigenètica transgeneracional a *Caenorhabditis elegans*, encara no ha estat descrit com l'exercici físic pot beneficiar els organismes a través de vies epigenètiques. En aquest estudi, implementem un tractament d'exercici físic a una població de *C. elegans*, i analitzem les generacions posteriors per estudiar els canvis hereditaris transgeneracionals en l'expressió gènica implicats en les vies relacionades amb l'envelliment. Hem posat èmfasi en la via del factor de transcripció CREB, el factor de transcripció de la resposta de creixement primerenca 1 (*EGR1*), la superòxid dismutasa 1 (*Sod1*) com a potent antioxidant i la via de proteïnes que conté dominis de desintegrina i metaloproteinasa (*ADAM10*) pel seu paper en el desenvolupament neurològic òptim.

Paraules clau: exercici físic, *C. elegans*, envelliment, epigenètica trangeneracional

Field integration

The current study is based on the main **pharmacology and therapeutics** field. Briefly, this experimental study aims to test how physical exercise might benefit organisms through epigenetic pathways in *C. elegans* model. Thus, it purposes to expand the knowledge of the molecular pathways involved in epigenetic processes in aging. Therefore, new therapeutic targets could be characterized and studied as a treatment for diseases where aging is the major risk factor, since current therapies have not demonstrated robust improvements in age-related decline.

Otherwise, the field **of biochemistry and molecular biology** is key in understanding the molecular pathways evaluated in the current study. The gene expression of proteins involved in more extensive and complex pathways has been analyzed such as CREB transcription factor or Early Growth Response 1 (ERG1) transcription factor, both related to a proper memory development. For this reason, this science allows us to reach a better understanding of molecular processes as well as a correct interpretation of the alteration of studied aging-related genes.

FInally, the older adult population has been increasing in recent decades and is expected to continue increasing worldwide. Moreover, aging is the main risk factor for most frequent human pathologies including cancer, cardiovascular disorders, and dementia, leading to a global **public health** matter. Although, even physical practice has been well demonstrated such as a preventive treatment against aging effects, physical inactivity also remains a major global public health problem.

Sustainable Development Goals (SDGs)

The current study is focused on *C elegans* nematode as a simplified organism to study aging processes in humans. Its short lifespan, human gene homology and well-known nervous system make this nematode a key experimental organism for transgenerational epigenetic research through aging decline process. Since the proportion of aging people is predicted to continue increase in follow decades, study of aging-related pathologies is a main health problem worldwide.

This project aims to expand the current knowledge of pathways involved in aging-related decline, more specifically, by evaluating the impact of long-term exercise practice in adult C. elegans and their offspring. Future study goals aim to discover deeply epigenetic regulation in aging-related pathways and its role in pathologies related to aging decline such neurodegeneration disorders. Moreover, new pharmacological targets could be described for the first time and contribute to new treatment and prevention lines.

Therefore, current study fits into the 2030 agenda for Sustainable Development adopted by United Nations member states in 2015, specifically in its third goal, which aims to ensure healthy lives and promote well-being for all at all ages, and its nineth goal that expects to build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation. Thus, the main objective of the study of aging and its associated pathologies is to extend knowledge to achieve a better quality of life at all ages. Moreover, investing in research contributes to the continuous innovation of knowledge, expanding methods and therapies that directly benefits public health.

INDEX

Introduction

Aging and neurological decline

Aging has been characterized as a gradual functional deterioration with a progressive physiological integrity loss, increasing the organism's vulnerability to death. This described decline is the principal risk factor for most frequent human pathologies including cancer, cardiovascular disorders, neurodegenerative diseases, and type 2 diabetes. [1,2]

Understanding of cellular mechanisms of aging-associated pathologies has progressed dramatically in the last decades. Therefore, the most important aging hallmarks have been related representing common denominators of organism deterioration (figure 1). These hallmarks are genomic instability, epigenetic alterations, telomere attrition, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication. [2]

Research suggests that each hallmark should be manifested during normal aging, it might sustain aggravation in accelerated aging process and its experimental amelioration should

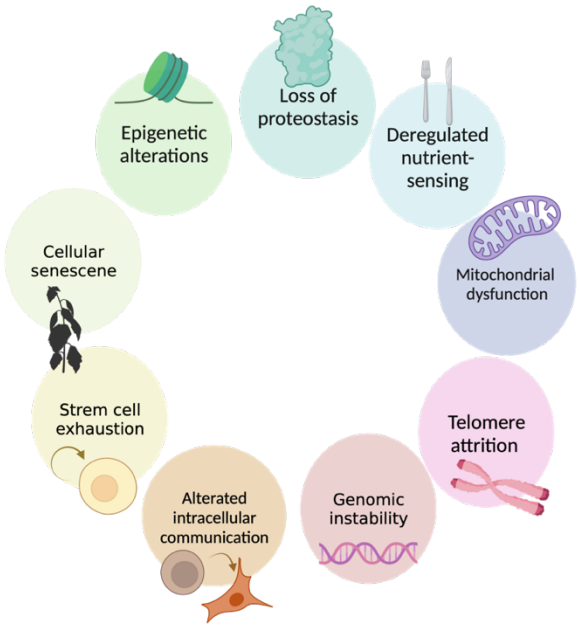

Figure 1: Diagram of aging hallmarks including loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, telomer attrition, genomic instability, intracellular communication, stem cell exhaustion, cellular senescence and epigenetic alterations.

delay normal aging increasing healthy lifespan. Accordingly, targeting those aging related pathways may contribute to the identification of new aging biomarkers. Therefore, potential pharmacological targets could we characterized by contributing to the understanding of biological basis of aging-related pathologies. [2,3]

Nowadays, one in three adults over 65 years of age dies with Alzheimer's disease (AD) or another related dementia [4]. Numerous risk factors are recognized to contribute to cognitive decline as genetic, socioeconomic, and environmental factors. Nevertheless, the main risk factor for dementia is advanced age. In the following decades, a rise in dementia cases is expected due to the increasing age of the world population. Therefore, it places neurodegeneration diseases as an alarming health problem worldwide. [3]

Currently, pharmacological therapies do not prove to significantly modify the course of age-associated cognitive impairment which underscores the crucial role of prevention. Recently, research has been crucial to define and correlate neurodegeneration hallmarks and molecular pathways, but there is still a gap in knowledge of mechanisms that lead the organism to dementia and its prevention remains poorly understood. [4,5]

Epigenetics and its role in aging

Aging is accompanied by a physiological decline and several major hallmarks, highlighting epigenetic alterations. Epigenetics represent reversible modifications that lead to genome expression regulation without alternating the DNA sequence. Therefore, epigenome connects genotype to phenotype by modulating the aging process in response to environmental factors. [6]

Epigenetic mechanisms could be simplified into three different modifications: DNA methylation, histone modification, and production of non-coding RNAs (figure 2). First, methylation of DNA is the covalent addition or subtraction of a methyl group to a DNA nucleotide. DNA methyltransferases controlled this process. Hypermethylation in promoter regions is associated to a repressed gene expression.

Histones are basic proteins of chromatin which act as spools around DNA winds. This structural histone/DNA unit is called nucleosome, a basic structural unit of DNA packaging. There are several types of histone modifications such as methylation and acetylation. These alterations can affect DNA-histone interactions leading to gene transcription alterations. In general, histone acetylation is associated with transcriptional activation while methylation can positively or negatively affect transcription. [7,8]

Non-codifying RNAs (ncRNAs) demonstrated to widely regulate gene expression in various biological processes at transcriptional and post-transcriptional level. Between all ncRNAs, micro-RNA (miRNA), an approximately 20 nucleotide long sequence capable of inhibiting protein formation by intercepting its corresponding mRNA play a relevant role in controlling gene expression. [9]

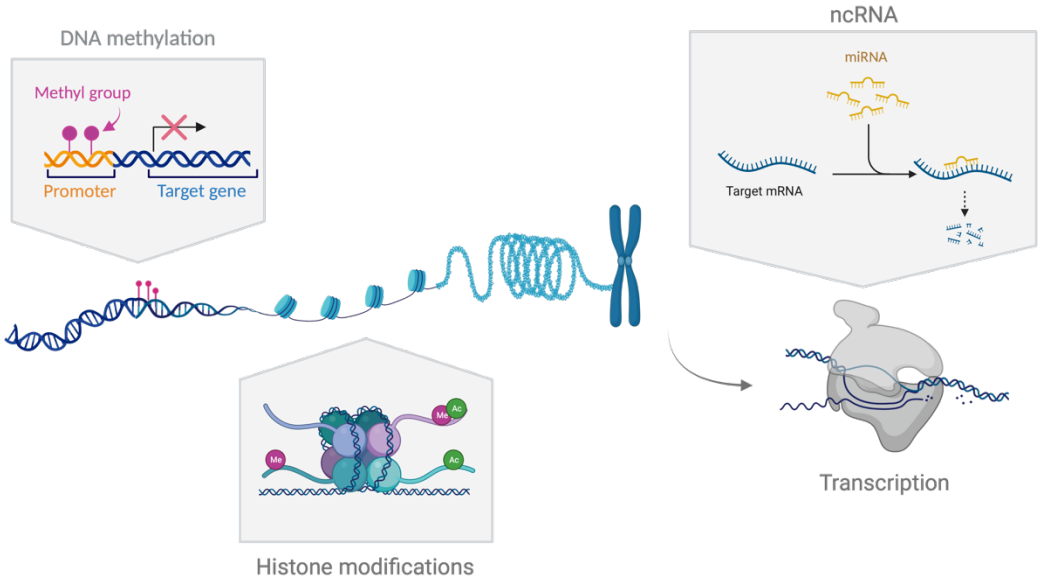

Figure 2: An overview of most common epigenetic modifications including DNA methylation, histone modification and ncRNA regulation.

It has been described that epigenetic modifications influenced by environment factors can be inherited to the subsequent generations and characterized as two different types.
Transgenerational inheritance is defined as any phenotypic change that persists for three or more generations. In contrast, intergenerational inheritance refers to epigenetic effects that only persists for a maximum of two more generations (figure 3). The origin of this distinction is conditioned by whether the genetic offspring material was present at the time of

environment factor exposure. Thus, intergenerational effects could be caused by the effects of the parent's environment exposure during the embryo, fetus, or gem cells formation. On the contrary, transgenerational effects could not be due to direct environment exposure.

Therefore, understanding the epigenetic mechanisms will contribute to characterize and develop new pharmacological strategies. Indeed, aging interventions based on epigenetic mechanisms have already led to lifespan extension in animal models. [10]

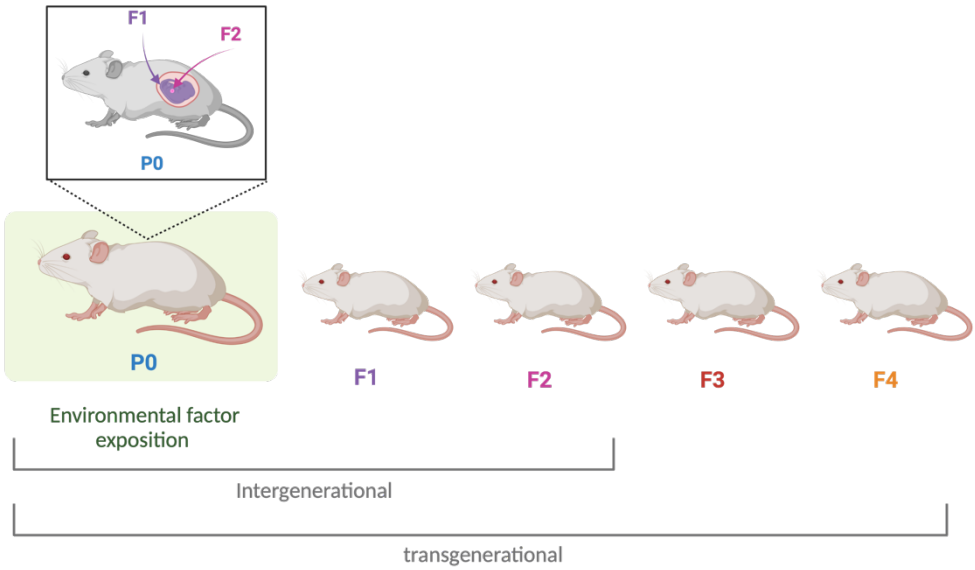

Figure 3: Graphic representation of intergenerational and transgenerational inheritance in mice.

Nonpharmacological interventions as a promising tool for aging disorders

Worldwide, the older adult population has been increasing in the last decades and is expected to continue incrementing over the next years concomitantly with the incidence of age-related diseases. Even though alternative non-pharmacological therapies as physical exercise and caloric restriction have proved promising benefits against aging and dementia, molecular mechanisms underlying these protections remain poorly understood, partially due to the time and cost investment of mammalian long-term studies. [11]

Healthy lifestyle is considered the most accessible prevention for aging decline. After studying long-term training in mice, animals experimented lifespan extension, learning ability benefits, and reduction of abnormal changes of the aged synapse. Even though physical practice has

been well demonstrated such as a preventive treatment to reverse the adverse effects of aging, physical inactivity remains a major global health problem (figure 4).

Physical activity has also proved to induce epigenetic modifications in organism. For instance, exercise may remodel DNA methylation in the promoter region of key genes in muscle. Moreover, histone modification and miRNAs regulation could be also induced by physical activity. [12]

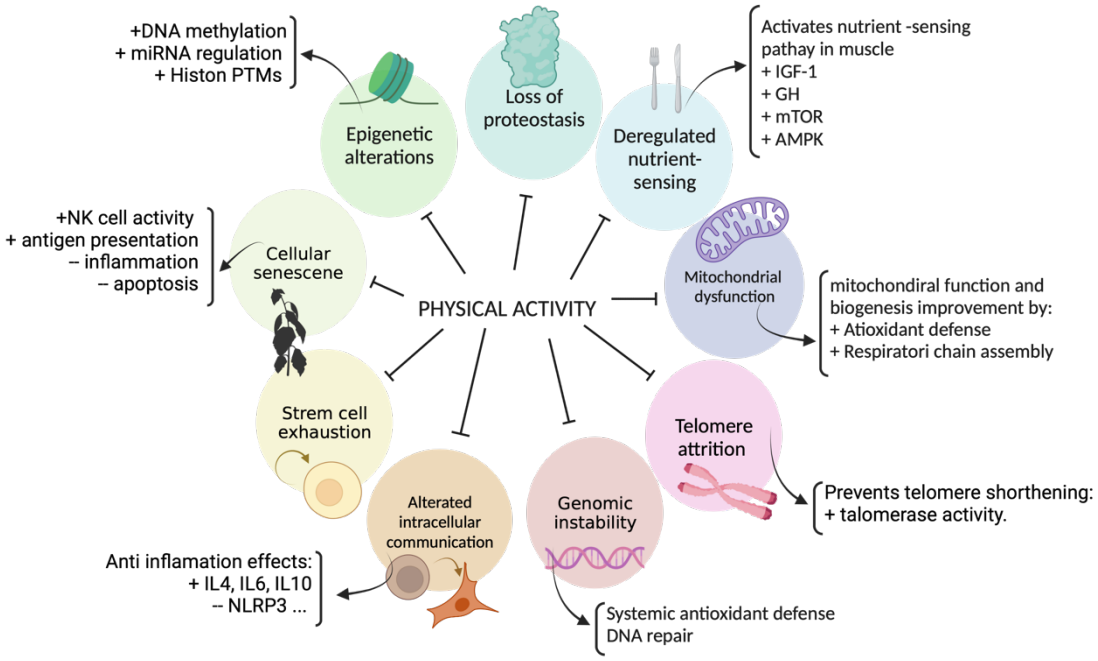

Figure 4: Summary of several anti-aging effects of regular exercise in aging hallmarks diagram.

Molecular pathways involved in memory, oxidative stress, and longevity

In the present study we focused on specific age-related pathways to analyze its gene expression. We emphasized on CREB transcription factor pathway, early growth response 1 (EGR1) transcription factor, superoxide dismutase 1 (Sod1) as a powerful antioxidant, and disintegrin and metalloproteinase domain-containing protein (ADAM10). Distant molecular pathways but all involved in aging decline.

The transcription factor cAMP response element binding-protein (CREB) has been related to cognitive function and neuronal excitability. Furthermore, CREB is required for long term associative memory but not for short-term memory. Research also points that the CREB expression declines with age alongside its activators and its partner intermediators. Thus, it became a potential target for the study of treatment cognitive age-related deficits. [13,14]

Immediate early genes (IEGs) are critical components in neuronal plasticity and neurotransmission integrating such complex interactions between environmental stimuli and gene expression. They supply the molecular framework for a dynamic response to neuronal activity while opening the possibility for a lasting adaptation by regulating of the expression of a wide range of genes. Therefore, the immediate early gene and transcription factor *early growth response 1* (*Erg1*) has been revealed as a major mediator and regulator of synaptic plasticity and neuronal activity in physiological and pathological conditions.[15]

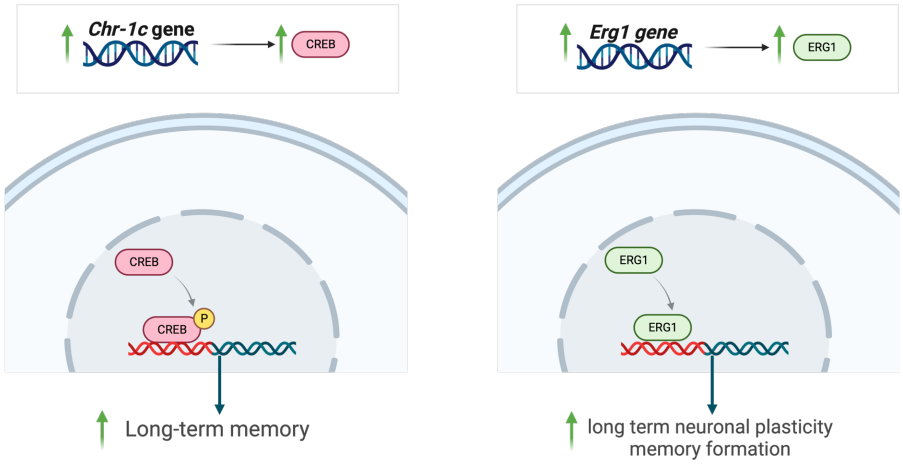

Figure 5: Simplified representation of CREB and ERG1 molecular pathways and their role in aging decline.

Several aging hallmarks are chronic inflammation, mitochondrial dysfunction and cellular senescence which occur progressively and irreversibly. Those aging hallmarks increase with oxidative stress. Superoxide dismutase 1(Sod1) serves as a major antioxidant in cell cytoplasm and neutralizes superoxide radicals throughout attenuating the age-related tissue changes and oxidative damage associated. [16]

α disintegrin and metalloproteinase 10 (ADAM10) is responsible for shedding of important proteins such as cadherins, ephrins, and Notch receptors where cleavage is required for

proper nervous system development. Furthermore, ADAM10 cleaves the amyloid precursor protein which prevents amyloid-β generation. Thus, ADAM10 has been described as an interesting enzyme in Alzheimer's disease (AD). [17]

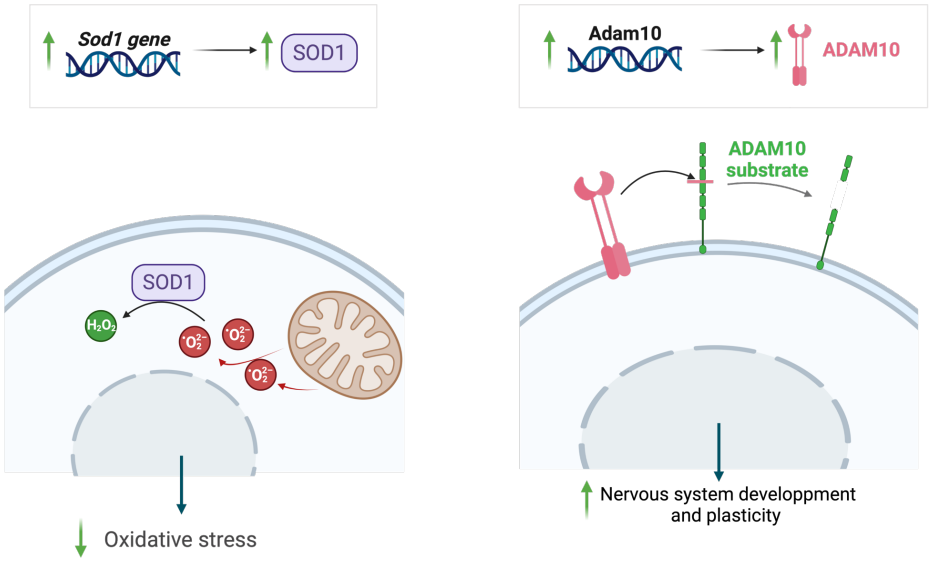

Figure 6: Simplified representation of SOD1 and ADAM10 molecular pathways and their role in aging decline.

Caenorhabditis elegans as a model used for the study of aging

The maximum lifespan differs between organisms from days to hundred of years in different species. Consequently, studies of human age-related diseases searched animal models with shorter lifespan and human gene homology. Thus, faster, and more robust results could be obtained while requiring less time and resources. [18]

Nowadays, much of our knowledge of aging genetics came from short-lived, non-vertebrate model organisms such as, worms, yeast, and flies as *Caenorhabditis elegans* or *Drosophila melanogaster.* Research suggests that many hallmarks of aging are preserved from yeast to humans through evolution. Furthermore, some of these conserved pathways, associated with human longevity, have been correlated with age-related diseases and they have a main role in aging studies. [19]

The *Caenorhabditis elegans* (*C. elegans*) nematode is an experimental model for primary neural research. Its transparent body, gene human homology, short lifespan, and its well-known nervous system, constitute a perfect model for the study of age-related pathways, cognitive function, and neurodegenerative diseases. [20]

C. elegans reach adulthood in approximately 3 days and its lifespan extends until 3 weeks. Short lifespan proved to be significantly advantageous for age-related studies. It permits to analyze aging markers over the entire adult life with considerable simple, economical, and fast methods. Moreover, nematode studies can involve many organisms which quickly permits to obtain robust statistically significant results. [20]

Nervous *C. elegans* system has been completely characterized. It is composed of 302 neurons in hermaphrodites and 385 in male adults. Thus, *C. elegans* is a perfect organism model for neurodegeneration studies. Considering its well-known nervous system alongside its short lifespan make this nematode a key experimental animal for transgenerational epigenetic research.

Furthermore, *C. elegans* genome has been studied searching homologies with human genes. When assayed, more than 40% of *C. elegans* protein-coding gens show orthology with human genome making this nematode valuable for genomic research [21].

Finally, the fully characterized musculature and the completely mapped nervous system of *C. elegans* could facilitate an analysis of the organism-wide effects of exercise. Importantly, characteristics of *C. elegans* muscle are conserved in humans. In addition, body wall muscle in *C. elegans* is the functional equivalent of vertebrate skeletal muscle. Its basic functional unit, the sarcomere, is highly conserved from nematodes to mammals through evolution in terms of composition, structure, and function.[22]

Objectives and hypothesis

Our central hypothesis is that physical exercise might induce better physical form in *C. elegans* adults as well as positive alteration of molecular pathways involved in healthy aging. Moreover, subsequent generations of treated *C. elegans* might inherite those molecular alterations via transgerational epigenetic regulation.

In the current study we aim to evaluate the influence in healthy aging of long-term exercise in *C. elegans* adults and their non-treated offspring. Firstly, to quantify the physical impact of long-term exercise practice by evaluating the body wall of *C. elegans* adults and their offspring. Furthermore, to evaluate nervous system condition through motility condition analysies of C*. elegans* adults treated with physical activity and their subsequent generations. Also, to study alterations in *C. elegans* cognitive development and memory formation by analyzing gene expression of *Chr1c* and *Ergh1* in treated adults and their offspring. Meanwhile, to evaluate oxidative stress response in C. elegans after long-term exercise practice through studying *Sod1* expression in treated adults and their descendants. Finally, to analyze the correct nervous system development of treated worms and their offspring via *Sup17* gene expression evaluation.

Additionally, the future goals of this set-up are focused on extrapolate this physical exercise treatment to other *C. elegans* strains used in neurodegenerative and cognitive function studies, like CL2006 strain as a model of Alzheimer's disease. Therefore, contributing to the rising of knowledge of those age-related neurodegeneration disorders with possible epigenetic targets not fully understood.

Materials and methods

Caenorhabditis elegans strains and maintenance

All worm strains used were maintained in Nematode Growth Medium (NGM) and stored at 16ºC. NGM agar is an enriched medium containing peptone, cholesterol, NaCl, $CaCl_2$, $MgSO_4$ and KPO_4. Plates were seeded with *E. coli* OP50 strain as a worm food source. OP50 strain is an uracil- requiring mutant used to prevent overgrowth of the bacterial layer. Due to limited uracil medium, restricted bacterial reproduction allowed easier observation and better worm mating. [23]

Two different C.*elegans* strains were used to carry out this experiment (figure 7). First, we selected the N2 strain as a wild type. N2 worms were used only for motility assays.

The second strain selected, PD4251 strain, was used in body wall muscle mass quantification processes. Furthermore, it was used to carry out molecular analyses. PD4251 express a GFP-myo-3 in vulval and body wall muscles. It integrates three important plasmids: pSAK2 (myo-3 promoter driving a nuclear-targeted GFP-LacZ fusion), pSAK4 (myo-3 promoter driving mitochondrially targeted GFP) and dpy-20 subclone. The dpy-20 gene encodes for a protein that is required for normal body morphology. [24]

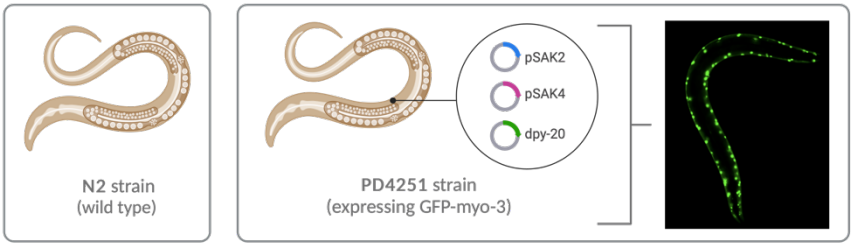

Figure 7: Diagram of *C.elegans* strains N2 and PD4251 used in the experiment

Long term swimming exercise

In the current study, swimming exercise treatment in adult *C. elegans* was implemented following a previously described protocol [25]. It established multiple daily swimming sessions that proved to increase muscle gene expression, locomotory function and multiple whole-animal parameters. Furthermore, it established that neurodegenerative pathologies in *C. elegans* models were ameliorated after swimming exercise treatment.

We scheduled a long-term exercise regimen in adult *C. elegans* based on 90' swimming sessions. The followed protocol proved its maximum efficiency when worms swam 3 sessions per day during the first two adulthood days plus 2 sessions per day in the following two days. We treated both strains (N2 and PD4251) and we performed three experiment repetitions.

Before starting the exercise regimen, worm synchronization was required to obtain a whole population of worms at a specific stage. To achieve that, we placed approximately 20 adult worms in a NGM seeded plate which was stored at 16ºC. After 4-6 hours we removed the *C. elegans* adults previously placed to obtain a full generation of synchronized eggs. This must be done 3 days before starting the swimming treatment since C. *elegans* eggs reach adulthood in 2-3 days at 16ºC.

Each swimming session started with previous 3 worm washes carried out with M9 buffer. Consequently, *E.coli* traces from NGM maintenance plate were removed. M9 is a physiological worm buffer enriched with KH_2PO_4, Na_2HPO_4, NaCl and $MgSO_4$. Thereafter, worms were transferred to a NGM unseeded plate and flooded with M9 buffer. Swimming plates were stored at 16ºC during the 90' swimming session. Afterwards, we extracted worms from NGM unseeded and flooded plate, we removed the M9 excess, and we relocate worms to their maintenance plate (figure 8).

Simultaneously, the control group followed the same procedure: 3 worm washes and worm transference to NGM unseeded plate. In contrast, control plates were not flooded with M9. Consequently, control group worms moved by crawling instead of swimming which is significatively less energetically demanding.

Finally, after the end of treatment, worm samples were collected just one day after the last session.

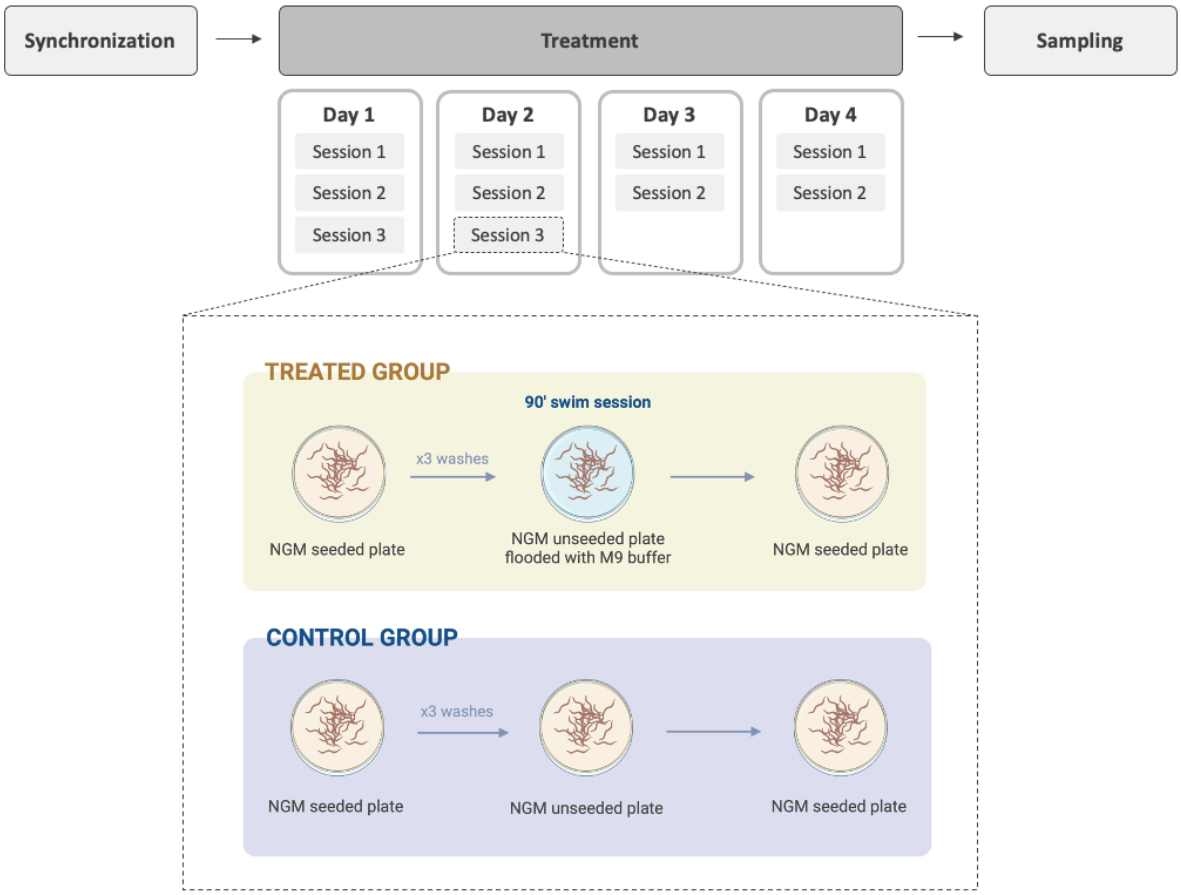

Figure 8: Schedule of long term swim exercise containing diagrams of procedures of control and treated groups. Only applicated to first generation worms (P0).

Maintenance and sampling of *C. elegans* offspring

In the current study, obtaining the descendants of the first treated generation was a key process. We used the synchronization procedure until de fifth generation. First, 20 worms from P0 generation were transferred to a new NGM seeded plate and removed after 4-6 hours. Three days after synchronization we obtained F1 adults (figure 9). We proceeded to sample them and repeated the process until the F5 generation. Plates were always stored at 16ºC.

Sampling process was carried out with previous three worm washes with the M9 buffer. Thereafter, M9 excess was discarded, and worm samples were stored at -20ºC for their preservation until molecular analyses.

Maintenance of worm's offspring was carried out in P0 treated group but also in P0 control group using both strains, N2 and PD4251.

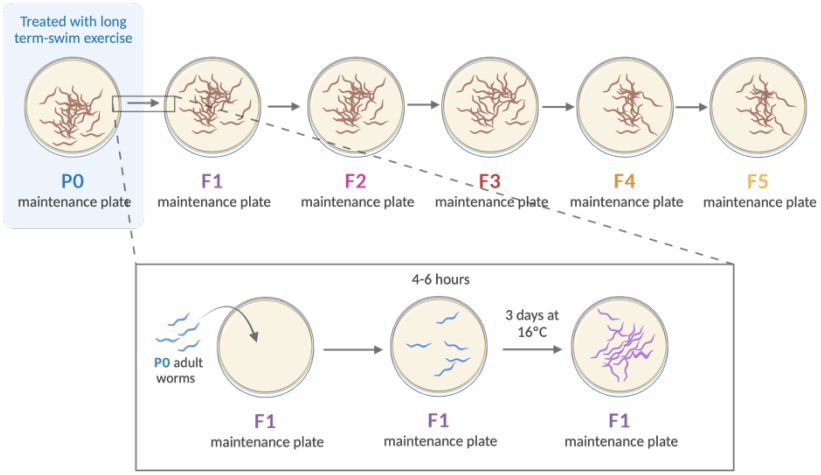

Figure 9: Diagram of the procedure used to obtain C.*elegans* offspring synchronized. Carried out in P0 control and P0 treated groups of N2 and PD4251 strains.

Quantification of body wall muscle mass

The day after finishing the long-term swimming exercise treatment we proceeded to quantify the body wall muscle mass in the PD4251 worms. We used PD4251 strain since it expresses a GFP-myo-3 in vulval and body wall muscles.

To quantify the body wall muscle, several worms of each group were photographed by fluorescence microscope capturing the GFP-myo-3. Thereafter, each worm picture was analyzed individually with the FIJI program. FIJI method allowed us to obtain a relative fluorescence value for each worm revealing its body wall muscle density. Approximately 20 worms were sampled for each group (control and treated) and for each generation (P0, F1, F2,F3, F4 and F5). The experiment was carried out in triplicate (figure 10).

Microscope slides were prepared using 2% liquid agarose as a worm base. After its solidification, 8 µL of 0,1% azide was added. Azide was used as a *C.elegans* paralyzing drug leading up to an efficient observation and worm photography. After slide preparation, sample worms were transferred to slides and kept in darkness until microscope observation.

Each body worm was pictured with an optical microscope using fluorescence and bright field modes. Florescence mode was used to detect the PD4251 GFP and bright field mode allowed us to observe worm orientation and morphology.

Finally, the microscope images were analyzed by selecting an image area representative of the individual worm's fluorescence. Caution was taken to select the same location and area in each worm for fluorescence calculation. Also, the mean background fluorescence was quantified corresponding to the same worm area previously analyzed. The final quantification of worm fluorescence was calculated correcting for background fluorescence.

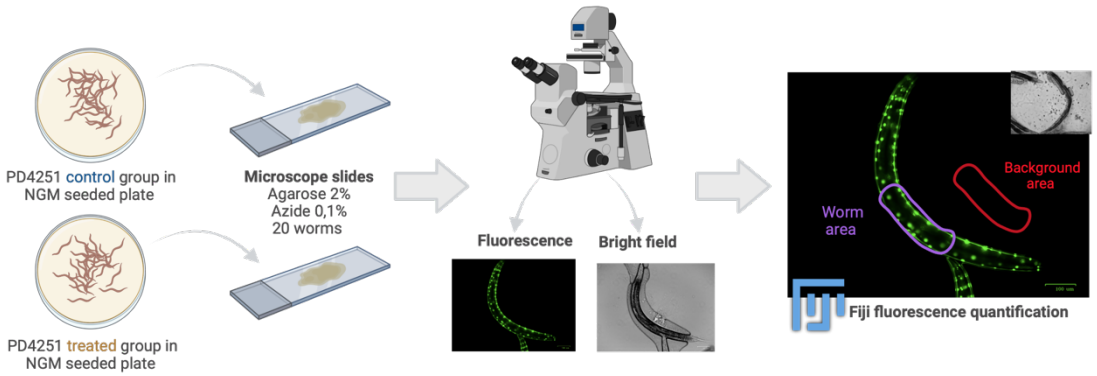

Figure 10: Simplified diagram of body wall muscle quantification process in PD4251 *C.elegans*. The procedure was executed in control and treated group for each generation (P0, F1, F2, F3,F4 and F5).

Mobility analysis through thrashing assay

The thrashing assay is a well-established method for evaluating mobility in *C. elegans***.** Nematodes are placed in liquid to estimate the frequency of lateral thrashing movements. It allows to assess the effects of chemicals or mutations on worm locomotion.

First, we prepared the microscope slides by adding a M9 drop. Immediately, one single worm was submerged into the buffer. We recorded each worm for a minute. We sampled 20 worms for each group (control and treated), for each generation (P0, F1, F2, F3, F4 and F5) and for each experiment's replicate. Motility tests were only evaluated in the N2 strain (figure 11).

Each worm video was analyzed individually by counting worm thrashes for one minute. The body angles through which the worms pass a "thrashing movement" were determined by the experimenter. Finally, we obtained Thrashes·min^{-1} values for each worm as a quantitative result directly correlated with worm motility.

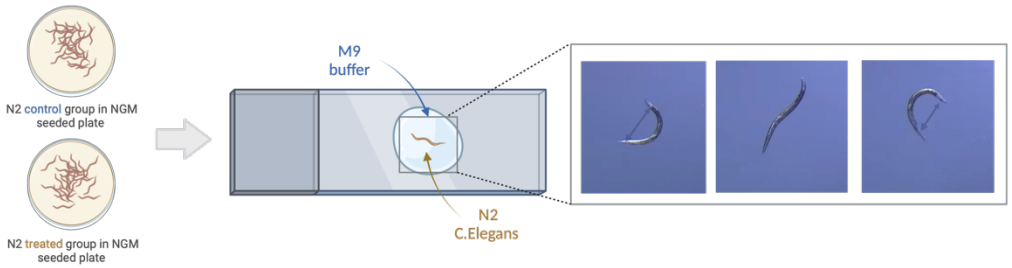

Figure 11. Graphic representation of thrashing assay preparation process in N2 *C.elegans*. The procedure was executed in control and treated group for each generation (P0, F1,F2,F3,F4 and F5).

RNA extraction and gene expression determination

The RNA isolation was carried out following a previously described protocol [26]. The RNA concentration was measured using a NanoDrop instrument. Acceptable results with 260/280 ratios were between 1.80 and 2.00. Furthermore, from the isolated mRNA, we obtained cDNA using the Trisure Bioline kit. Afterwards, reverse transcription polymerase chain reaction was performed by employing the High-Capacity cDNA Reverse Transcription kit (Applied

BiosystemsTM) treating cDNA samples with the specific gene primers. In the current study primers used were *Chr1c*, *Ergh1*, *Sod1* and *Sup17*. Also, actin (*act*) was analyzed as the housekeeping gene. Finally, we calculated relative expression using the cycle threshold method (ΔΔCt) normalized with actin gene levels. Each sample was analyzed in duplicate.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism. All data groups were tested with Iterative Grubbs' method to identify data outliers considering alpha=0.1. The statistical outliers detected were removed. Unpaired two-tailed t-test was used to compare the two data groups (control and treated) for each generation. Statistical significance was considered when $p<0.05$.

Results

Long-term swimming exercise increases body wall muscle mass in adult *C. elegans* and their subsequent generations

We treated P0 adults with multiple daily swimming sessions following the protocol previously described. Then, only the first generation was treated. Subsequently, we took adults' samples of four more generations to analyze whether some improvements in body wall muscle mass could be inherited transgenerationally. Simultaneously, we carried out a control group throughout all the generations.

To quantify muscle mass we used a transgenic *C.elegans* PD4251 strain, expressing GFP-myo-3 localized in vulval and body wall muscles. We sampled 20 worms approximately for each group and we quantified the fluorescence intensity for each worm. Three experiment repetitions were carried out. Finally, we performed statistical analyses for each generation to compare treated and control group.

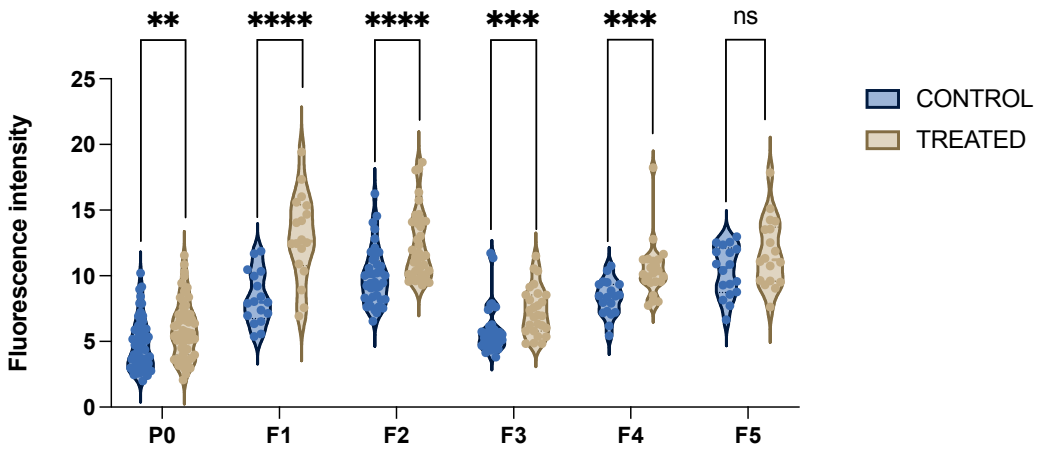

Figure 12: Distribution of the two groups, control and treated and their offspring until de fifth generation. Considering its body wall muscle fluorescence due to GFP- myo-3. The data of three experiment replications were considered and grouped.
P0: p=0.0031**, n=115. Difference between means (Treated-Control) ± SEM = 1.173 ± 0.3886.
F1: p<0.0001****, n=38. Difference between means (Treated-Control) ± SEM = 4,495 ± 0,8721.
F2: p<0.0001****, n=78. Difference between means (Treated-Control) ± SEM = 2.501 ± 0.5205.
F3: p<0.0003***, n=60. Difference between means (Treated-Control) ± SEM = 1.639 ± 0.4292.
F4: p<0.0002***, n=40. Difference between means (Treated-Control) ± SEM = 1.771 ± 0.4211.
F5: p<0.0699 (ns), n=36. Difference between means (Treated-Control) ± SEM = 1.419 ± 0.7584.

A significant increase in body wall muscle was detected in the treated group but also in the four subsequent generations, suggesting that the beneficial effects of exercise were inherited opening the opportunity to evaluate epigenetics due to the F3 generations without direct stimulus from exercise presenting the same pattern as P0 or F1. Statistical significance was lost in the fifth generation, suggesting the participation of microRNAs in this process (figure 12).

Long-term swimming exercise increases mobility in adult *C. elegans* but does not suggest inherited modifications in subsequent generations

After exercise treatment, an evaluation of worm mobility was carried out based on the thrashing assay to check synaptic dysfunction [27]. We sampled approximately 20 worms per group and for each generation. Each worm was analyzed individually to determine the number of thrashes per minute. Afterward, we performed three experiment repetitions, and we grouped the data. Finally, statistical analyses were performed in each generation comparing control and treated groups.

Although significance was only obtained in the first generation (P0) from F1 to F4 we showed the same pattern, indicating the necessity to increase samples to obtaining statistical significance in those groups (figure 13).

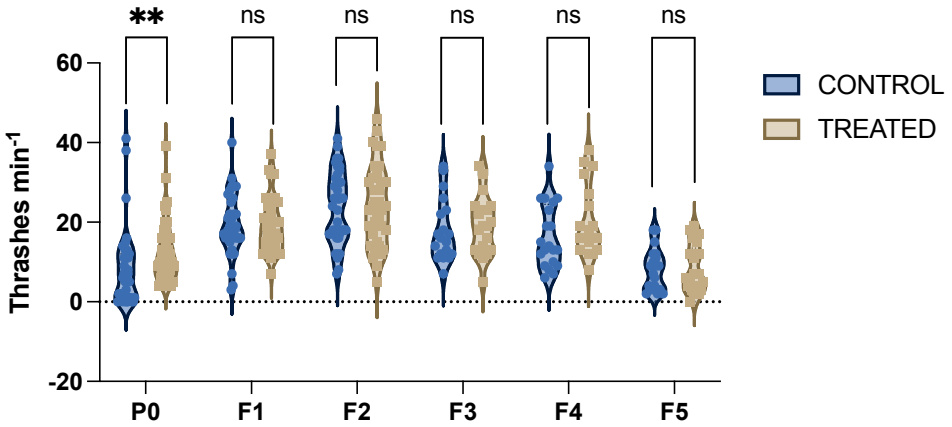

Figure 13. Distribution of worm mobility classified by control and treated group in six generations. Worm mobility is expressed as thrashes per minute. The data of three experiment replications were considered and grouped. **P0**: p=0.0090 **, n=78. Difference between means (Treated-Control) ± SEM = 4.359 ± 1.626.

Long-term exercise treatment influences gene expression of several genes in worm offspring

PD4251 strain was used to carry out molecular assays. mRNA of each group sample was extracted and analyzed by qPCR to quantify gene expression involved in cognitive function, oxidative stress, and neuroprotection.

The first gene evaluated, *Chr1c* gene, is involved in long term associative memory processes. Its human homolog *Chr1c* expression was not modified in the first generation subject to exercise treatment.

However, F2 generation showed a significant upregulation of *Chr1c* expression in the treated offspring. None of the other generations showed relevant gene expression modifications that could be attributed to exercise treatment (figure 14.A).

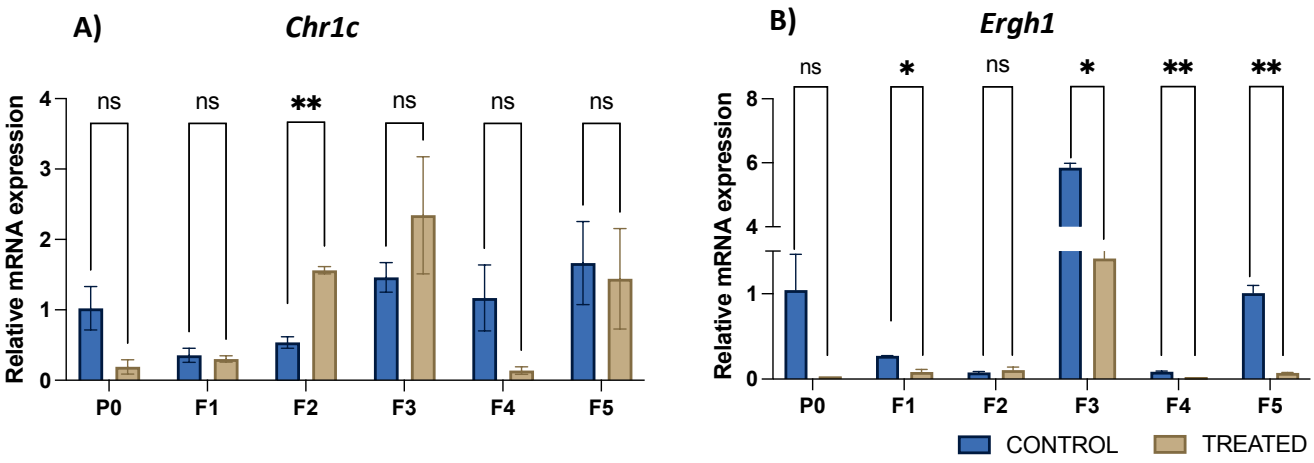

Figure 14. Relative mRNA expression comparing treated offspring of worms exposed to long-term swim exercise treatment and their respective controls. Each group was analyzed in duplicated. **A)** Relative *Chr1c* mRNA expression. **F2**: p=0.0045**, difference between means (Treated-Control) ± SEM = 1.026 ± 0.06936. **B)** Relative *Ergh1* mRNA expression.
F1: p=0.0121*, difference between means (Treated-Control) ± SEM = -0,1910 ± 0,02121.
F3: p=0.0131 *, difference between means (Treated-Control) ± SEM = -4,434 ± 0,5129.
F4: p=0.0061**, difference between means (Treated-Control) ± SEM = -0,06875 ± 0,005384.
F5: p=0.0046**, difference between means (Treated-Control) ± SEM = -0,9357 ± 0,06398

An additional gene related to memory processes was analyzed. *Ergh1* gene is the *C. elegans* homolog of *Erg1* in humans. *Erg1* induction has been shown to have an important role in neuronal plasticity and memory pathways, concomitant to our results obtained in the thrashing assay.

In the current study exercise treatment downregulates significantly *Ergh1* expression in the subsequent F1, F3, F4 and F5 generations. Of note, exercise does not show significant gene expression alterations in the first generation (P0) (figure 14.B).

Moreover, the *Sod1* gene was studied to evaluate the antioxidant response. It codifies superoxide dismutase enzyme, implicated in antioxidant response against reactive oxygen species. Also, the presence of ROS has been associated with aging, and increased expression of superoxide dismutase has been demonstrate to lengthen lifespan and longevity in *C.elegans*. [28]

After swimming treatment in P0 worms, *Sod1* expression was downregulated significantly after four generations, in F4 and F5. Conversely, its expression does not show significant alterations in previously generations (figure 15.A).

Finally, *Sup17* expression was evaluated in worms. The human homolog of the *C. elegans Sup17* gene is *Adam10*. It codifies for a disintegrin and metalloproteinase domain-containing protein also called ADAM10. Its expression is associated to a correct neurodevelopment. Consequently, an alteration of ADAM10 activity correlates to neurodevelopmental disorders. [29]

Here, worms from P0 generation, treated with long-term swimming exercise showed a downregulation of *Sup17* expression. Furthermore, relative *Sup17* mRNA expression decreased significantly in treated offspring worms in F4 and F5 generations compared to their respective control groups (figure 15.B).

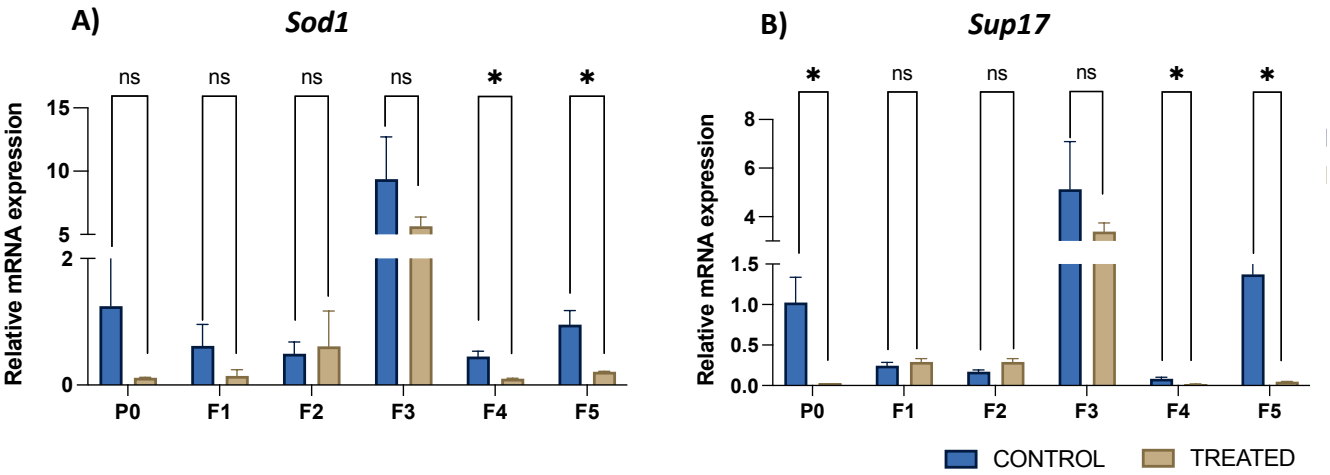

Figure 15. Relative mRNA expression comparing treated offspring of worms exposed to long-term swim exercise treatment and their respective controls. Each group was analyzed in duplicated. **A) Relative *Sod1* mRNA expression. F4**: p=0.0275*, difference between means (Treated-Control) ± SEM = -0,3518 ± 0,05954. F5: p=0.0416*, difference between means (Treated-Control) ± SEM = -0,7441 ± 0,1567. **B) Relative *Sup17* mRNA expression. P0**: p=0.0459*, difference between means (Treated-Control) ± SEM = -0,9957 ± 0,2211. **F4**: p=0.0335*, difference between means (Treated-Control) ± SEM = -0,06960 ± 0,01306. **F5**: p=0.0070**, difference between means (Treated-Control) ± SEM = -1,324 ± 0,1113.

Discussion

Long-term swim exercise delays body wall muscle decline *in C. elegans* adults. The treated group showed a significantly more dense wall muscle mass than the non-treated group indicating that this swim exercise intervention works properly to *C.elegans*. Moreover, significant increase of body wall muscle mass was also observed in subsequent fourth generations.

The improvements observed in body muscle reinforce the hypothesis that physical exercise provide general health benefits against aging-decline in muscle and could be proposed as a preventing treatment of age-related pathologies and cognitive decline. Furthermore, the muscle improvements quantified in subsequent generations suggests that physical activity might induce epigenetic transgenerational modifications in muscle. Future study goals could aim to analyze epigenetic biomarkers in *C. elegans* muscle after long-term exercise treatment to expand knowledge of epigenetic mechanisms stimulated by physical activity.

After exercise treatment, worm mobility increased significantly in the treated group suggesting an enhance in age-related synaptic dysfunction. Treated *C. elegans* offspring showed the same mobility pattern in control and treated offspring without any significant variation, indicating the requirement to increase sample size to obtain statistical significance in those groups. Implementation of automated thrashing frequency counting programs could replace the far more time-consuming manual method, contributing to the expansion of thrashing samples.

Significant increase of *Chr1c* gene expression in F2 nematodes, after a long-term physical practice implemented in P0 generation, evidence that exercise upregulates the expression of CREB transcription factor and suggests the involvement of epigenetic intergenerational mechanisms due to direct exposure to exercise since this regulation shows to be lost in the F3 generation. Consequently, long-term associative memory, cognitive performance, and neuronal excitability processes should improve in the exercised *C.elegans* group and its descendants. Further studies are needed to carry out focusing on the influence of this long-term

exercise on cognition and memory. The use of long-term memory tests, and the evaluation of epigenetic mechanisms implicated might be evaluated in future studies.

Erg1 belongs to the category of immediate early genes (IEG) since it modulates the expression of many genes. It codifies for a transcription factor protein (ERG1) which has a particular expression pattern in the brain which has been related to plasticity-linked function in neuronal system and memory development. In the current study, exercise-treated adults offspring downregulates significantly *Ergh1* expression in F1, F3, F4 and F5. This downregulation across six generations supports that ERG1 expression is subject to exercise-stimulated epigenetic regulation. Despite the robust statistical signification in results, literature reaffirms that physical activity down-regulates *Erg1* expression by promoting a proper brain plasticity.

Antioxidant response was evaluated through the study of *Sod1* gene expression. Since, the presence of ROS has been associated with aging, and increased expression of superoxide dismutase has been demonstrate to lengthen lifespan and longevity in *C.elegans*. Although, in the current study physical activity practice induced a significant downregulation of *Sod1* expression in treated adult's offspring suggesting the implication of epigenetic regulation and the transgenerational inheritance processes.

Finally, *Sup17* expression was evaluated in worms associated to a correct neurodevelopment since it codifies for a disintegrin and metalloproteinase domain-containing protein also called ADAM10 associated to a correct neurodevelopment and highly related to AD. *C. elegans* treated with long-term swimming exercise showed a downregulation in *Sup17* expression. This significantly decreased expression is also observed in F4 and F5 generations suggesting epigenetic modulation into ADAM10 pathway and its implication to nervous development. Despite, literature supports a upregulation of this disintegrin and metalloproteinase protein though exercise practice and associates ADAM10 alterations to serious neurodegeneration disorders.

In this experiment we demonstrated a significant alteration of gene expression of various age-related genes caused by long-term physical activity across six generations in *C.elegans*. This study supports the improvement of physical condition due to exercise in the aging process.

Besties, some of molecular analyses results seems to be contradictory to literature. In the future, a better analysis of the molecular pathways studied must be carried out. Moreover, the possibility of analyzing other pathways or intermediaries related to aging must be considered. Furthermore, it is necessary to increase the robustness of the molecular analyses by increasing the number of repetitions and by studying the gene expression also in the wild-type *C.elegans* strain since transgenic strains of *C.elegans* could mask certain molecular analyses of gene expression.

Of note, there is a lot of research needed after this set-up study because of the mechanisms by which we obtained inheritance across generations in *C.elegans* remain elucidate exactly. In the future, long term-swim exercise should be implemented in *C. elegans* strains used as neurodegeneration models to study neurodegeneration disorders and their epigenetic regulation mechanisms to search for new pharmacological targets that could be described for the first time.

.

Conclusion

In the current study, we demonstrated that physical exercise induces better physical form in *C. elegans* adults as well as positive alteration in their offspring physical condition suggesting transgerational inheritance patterns. Moreover, molecular analyses also proved significant alterations of gene expression in treated descendants supporting *C. elegans* might inherit those molecular alterations via transgerational epigenetic regulation.

Long term swim exercise improved body wall muscle mass in *C. elegans* adults and their non-treated offspring. Furthermore, the evaluation of nervous system condition through motility analyses of C*. elegans* adults treated with physical activity and their offspring only demonstrates significant motility improvements in first generation treated. Moreover, to study alterations in *C. elegans* cognitive development and memory formation by analyzing gene expression of *Chr1c* and *Ergh1*, the evaluation of oxidative stress response through studying *Sod1* expression, and the examination of nervous system development via *Sup17* gene expression, all supports that physical exercise-induced transgenerational epigenetic regulations. Even though, the limited results' robustness does not allow us to define these elements' role in aging decline processes.

References

1. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013 Jun 6;153(6):1194-217. doi: 10.1016/j.cell.2013.05.039. PMID: 23746838; PMCID: PMC3836174.
2. McHugh D, Gil J. Senescence and aging: Causes, consequences, and therapeutic avenues. J Cell Biol. 2018 Jan 2;217(1):65-77. doi: 10.1083/jcb.201708092. Epub 2017 Nov 7. PMID: 29114066; PMCID: PMC5748990.
3. Hou Y, Dan X, Babbar M, Wei Y, Hasselbalch SG, Croteau DL, Bohr VA. Ageing as a risk factor for neurodegenerative disease. Nat Rev Neurol. 2019 Oct;15(10):565-581. doi: 10.1038/s41582-019-0244-7. Epub 2019 Sep 9. PMID: 31501588.
4. Barnes JN. Exercise, cognitive function, and aging. Adv Physiol Educ. 2015 Jun;39(2):55- 62. doi: 10.1152/advan.00101.2014. PMID: 26031719; PMCID: PMC4587595.
5. Liu RM. Aging, Cellular Senescence, and Alzheimer's Disease. Int J Mol Sci. 2022 Feb 11;23(4):1989. doi: 10.3390/ijms23041989. PMID: 35216123; PMCID: PMC8874507.
6. (Wang K, Liu H, Hu Q, Wang L, Liu J, Zheng Z, Zhang W, Ren J, Zhu F, Liu GH. Epigenetic regulation of aging: implications for interventions of aging and diseases. Signal Transduct Target Ther. 2022 Nov 7;7(1):374. doi: 10.1038/s41392-022-01211-8. PMID: 36336680; PMCID: PMC9637765.)
7. Burton NO, Greer EL. Multigenerational epigenetic inheritance: Transmitting information across generations. Semin Cell Dev Biol. 2022 Jul;127:121-132. doi: 10.1016/j.semcdb.2021.08.006. Epub 2021 Aug 21. PMID: 34426067; PMCID: PMC8858334.
8. Hamilton JP. Epigenetics: principles and practice. Dig Dis. 2011;29(2):130-5. doi: 10.1159/000323874. Epub 2011 Jul 5. PMID: 21734376; PMCID: PMC3134032.
9. Wang K, Liu H, Hu Q, Wang L, Liu J, Zheng Z, Zhang W, Ren J, Zhu F, Liu GH. Epigenetic regulation of aging: implications for interventions of aging and diseases. Signal Transduct Target Ther. 2022 Nov 7;7(1):374. doi: 10.1038/s41392-022-01211-8. PMID: 36336680; PMCID: PMC9637765.
10. Greer EL, Maures TJ, Hauswirth AG, Green EM, Leeman DS, Maro GS, Han S, Banko MR, Gozani O, Brunet A. Members of the H3K4 trimethylation complex regulate

lifespan in a germline-dependent manner in C. elegans. Nature. 2010 Jul 15;466(7304):383-7. doi: 10.1038/nature09195. Epub 2010 Jun 16. PMID: 20555324; PMCID: PMC3075006.

11. Alty J, Farrow M, Lawler K. Exercise and dementia prevention. Pract Neurol. 2020 May;20(3):234-240. doi: 10.1136/practneurol-2019-002335. Epub 2020 Jan 21. PMID: 31964800.
12. Hartman JH, Smith LL, Gordon KL, Laranjeiro R, Driscoll M, Sherwood DR, Meyer JN. Swimming Exercise and Transient Food Deprivation in Caenorhabditis elegans Promote Mitochondrial Maintenance and Protect Against Chemical-Induced Mitotoxicity. Sci Rep. 2018 May 29;8(1):8359. doi: 10.1038/s41598-018-26552-9. PMID: 29844465; PMCID: PMC5974391
13. Kauffman AL, Ashraf JM, Corces-Zimmerman MR, Landis JN, Murphy CT. Insulin signaling and dietary restriction differentially influence the decline of learning and memory with age. PLoS Biol. 2010 May 18;8(5):e1000372. doi: 10.1371/journal.pbio.1000372. PMID: 20502519; PMCID: PMC2872642
14. Yu XW, Oh MM, Disterhoft JF. CREB, cellular excitability, and cognition: Implications for aging. Behav Brain Res. 2017 Mar 30;322(Pt B):206-211. doi: 10.1016/j.bbr.2016.07.042. Epub 2016 Jul 28. PMID: 27478142; PMCID: PMC5274584
15. Duclot F, Kabbaj M. The Role of Early Growth Response 1 (EGR1) in Brain Plasticity and Neuropsychiatric Disorders. Front Behav Neurosci. 2017 Mar 6;11:35. doi: 10.3389/fnbeh.2017.00035. PMID: 28321184; PMCID: PMC5337695.
16. Watanabe K, Shibuya S, Ozawa Y, Nojiri H, Izuo N, Yokote K, Shimizu T. Superoxide dismutase 1 loss disturbs intracellular redox signaling, resulting in global age-related pathological changes. Biomed Res Int. 2014;2014:140165. doi: 10.1155/2014/140165. Epub 2014 Sep 8. PMID: 25276767; PMCID: PMC4170698.
17. Elsworthy RJ, Hill EJ, Dunleavy C, Aldred S. The role of ADAM10 in astrocytes: Implications for Alzheimer's disease. Front Aging Neurosci. 2022 Nov 30;14:1056507. doi: 10.3389/fnagi.2022.1056507. PMID: 36533182; PMCID: PMC9748279.
18. Mitchell SJ, Scheibye-Knudsen M, Longo DL, de Cabo R. Animal models of aging research: implications for human aging and age-related diseases. Annu Rev Anim Biosci. 2015;3:283-303. doi: 10.1146/annurev-animal-022114-110829. PMID: 25689319.

19. Singh PP, Demmitt BA, Nath RD, Brunet A. The Genetics of Aging: A Vertebrate Perspective. Cell. 2019 Mar 21;177(1):200-220. doi: 10.1016/j.cell.2019.02.038. PMID: 30901541; PMCID: PMC7592626.
20. R. Laranjeiro, G. Harinath, D. Burke, B. P. Braeckman, M. Driscoll, Single swim sessions in C. elegans induce key features of mammalian exercise. BMC Biol. 15, 30 (2017).
21. Shaye DD, Greenwald I. OrthoList: a compendium of C. elegans genes with human orthologs [published correction appears in PLoS One. 2014;9(1). doi:10.1371/annotation/f5ffb738-a176-4a43-b0e0-249cdea45fe0]. PLoS One. 2011;6(5):e20085. doi:10.1371/journal.pone.0020085
22. Gieseler K, Qadota H, Benian GM. Development, structure, and maintenance of C. elegans body wall muscle. In: WormBook: The Online Review of C. elegans Biology [Internet]. Pasadena (CA): WormBook; 2005-2018. Available from: https://www.ncbi.nlm.nih.gov/books/NBK426064/
23. Brenner S. The genetics of *Caenorhabditis elegans*. Genetics. 1974 May;77(1):71-94. doi: 10.1093/genetics/77.1.71. PMID: 4366476; PMCID: PMC1213120.
24. Gene - WormBase : Nematode information resource [Internet]. Wormbase.org. [citated in 11/05/2022]. Available: https://wormbase.org/species/c_elegans/gene
25. Laranjeiro R, Harinath G, Hewitt JE, Hartman JH, Royal MA, Meyer JN, Vanapalli SA, Driscoll M. Swim exercise in Caenorhabditis elegansextends neuromuscular and gut healthspan, enhances learning ability, and protects against neurodegeneration. Proc Natl Acad Sci U S A. 2019 Nov 19;116(47):23829-23839. doi: 10.1073/pnas.1909210116. Epub 2019 Nov 4. PMID: 31685639; PMCID: PMC6876156.
26. Griñán-Ferré C, Bellver-Sanchis A, Olivares-Martín M, Bañuelos-Hortigüela O, Pallàs M. Synergistic Neuroprotective Effects of a Natural Product Mixture against AD Hallmarks and Cognitive Decline in Caenorhabditis elegans and an SAMP8 Mice Model. Nutrients. 2021 Jul 14;13(7):2411. doi: 10.3390/nu13072411. PMID: 34371921; PMCID: PMC8308558.
27. Dubey AA, Krygier M, Szulc NA, Rutkowska K, Kosińska J, Pollak A, Rydzanicz M, Kmieć T, Mazurkiewicz-Bełdzińska M, Pokrzywa W, Płoski R. A novel de novo FEM1C variant is linked to neurodevelopmental disorder with absent speech, pyramidal signs and limb ataxia. Hum Mol Genet. 2023 Mar 20;32(7):1152-1161. doi: 10.1093/hmg/ddac276. PMID: 36336956; PMCID: PMC10026218.

28. Melov S, Ravenscroft J, Malik S, Gill MS, Walker DW, Clayton PE, Wallace DC, Malfroy B, Doctrow SR, Lithgow GJ. Extension of life-span with superoxide dismutase/catalase mimetics. Science. 2000 Sep 1;289(5484):1567-9. doi: 10.1126/science.289.5484.1567. PMID: 10968795.
29. Marcello E, Borroni B, Pelucchi S, Gardoni F, Di Luca M (November 2017). "ADAM10 as a therapeutic target for brain diseases: from developmental disorders to Alzheimer's disease". Expert Opinion on Therapeutic Targets. 21 (11): 1017–1026.